



The interaction of bacteria with volcanic rocks on Earth and in space

Bo Byloos

Promotors

Prof. Dr. Ir. Nico Boon

Center for Microbial Ecology and Technology (CMET), Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium

Dr. Ir. Natalie Leys

Microbiology Unit, Interdisciplinary Biosciences, Institute for Environment, Health and Safety, SCK•CEN, Mol, Belgium

Dr. Ir. Rob Van Houdt

Microbiology Unit, Interdisciplinary Biosciences, Institute for Environment, Health and Safety, SCK•CEN, Mol, Belgium

Members of the examination committee:

Chair: Prof. Dr. Ir. Monica Höfte, Laboratory of Phytopathology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium

Secretary: Prof. Dr. Danny Geelen, In Vitro Biology and Horticulture, Department Plant Production, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium

Prof. Dr. Ir. Tom Hennebel, Center for Microbial Ecology and Technology (CMET), Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium

Prof. Dr. Aurélien Carlier, LM-UGent: Laboratory of Microbiology, Department Biochemistry and Microbiology, Faculty of Sciences, Ghent University, Gent, Belgium

Prof. Dr. Veerle Cnudde, UGCT - Centre for X-ray Tomography, Departement of Geology, Faculty of Sciences, Ghent University, Gent, Belgium

Prof. Dr. Ir. Rudy Swennen, Division of Geology, Department of Earth and Environmental Sciences, Faculty of Science, University of Leuven, Leuven, Belgium

Prof. Dr. Kai Finster, Department of Bioscience-Microbiology, Faculty of Science and Technology, University of Aarhus, Aarhus, Denmark

Dean Faculty bioscience engineering

Prof. dr. ir. Marc Van Meirvenne

Rector Ghent University

Prof dr. Anne De Paepe



The interaction of bacteria with volcanic rocks on Earth and in Space

Bo Byloos

**Thesis submitted in fulfillment of the requirements for the degree of Doctor
(PhD) of Applied Biological Sciences**

Promotors:

Prof. Dr. Ir. Nico Boon

Center for Microbial Ecology and Technology, Ghent University, Gent, Belgium

Dr. Ir. Natalie Leys

Microbiology Unit, SCK•CEN, Mol, Belgium

Dr. Ir. Rob Van Houdt

Microbiology Unit, SCK•CEN, Mol, Belgium

Titel van het doctoraat in het Nederlands:

De interacties van bacteriën met vulkanisch gesteente op Aarde en in de ruimte

Cover illustration:

Photo taken at the Námafjall, Hverarönd hot springs in Iceland during the Erasmus Mundus training program on 'Arctic Microbiology'

Funding:

This work was supported by the European Space Agency (ESA-PRODEX), Belgian Science Policy (BELSPO) through the E-GEM/BioRock project and the Inter-University Attraction Pole (IUAP) “ μ -manager” funded by the Belgian Science Policy (BE, 305 P7/25)

ISBN : 978-94-6357-024-4

Please refer to this work as: Byloos, B. 2017. The interaction of bacteria with volcanic rocks on Earth and in space. PhD thesis, Ghent University, Ghent, Belgium, p. 260

The author and the promoters give the authorisation to consult and to copy parts of this work for personal use only. Every other use is subject to the copyright laws. Permission to reproduce any material contained in this work should be obtained from the author.

*We began as wanderers and we are wanderers still
We have lingered long enough on the shores of the cosmic ocean
We are at last ready to set sail for the stars.*
Carl Sagan

Science is organized knowledge. Wisdom is organized life.
Immanuel Kant

*If you are happy with the limits of your own imagination, you will
never reach your full potential. With others, the limits will continue to
expand.*
Christoph Waltz

Notation index

AMO	Abundance Matched OTU
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCECF-AM	20,70-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-AM
BCM	Bacterial Controlled Mineralization
BIM	Biologically Induced Mineralization
CELSS	Controlled Ecological Life Support System
cF	cFluorescein
cFDA	5(6)-Carboxyfluorescein diacetate
cFDA-AM	Carboxyfluorescein Diacetate Acetoxymethyl Ester
cFDA-SE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFU	Colony Forming Unit
COSPAR	Committee on Space Research
CTAB	Cetyl Trimethylammonium Bromide
CTC	Citoyl Tetrazolium Chloride
CV	Crystal Violet
DAP	Diaminopimelic Acid
DIBAC ₄ (3)	Bis(1,3-dibutylbarbituric acid) Trimethine Oxonol
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances
ESA	European Space Agency
FC	Flow Cytometry
FSC	Forward Scatter
GSH	Non-Enzymatic Anti-Oxidants Like Glutathione
HDMS	Hexamethyldisilazane
HE	Dihydroethidium (hydroethidine)
HOMOVA	Homogeneity of Molecular Variance
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ISRU	In Situ Resource Utilisation
ISS	International Space Station
ISSym	Identity Sequence Similarity
KREEP	Potassium (K), Rare Earth Elements (REE), and Phosphorus (P) rocks
LB	Lysogeny Broth
LOI	Loss on ignition
MELISSA	European Micro Ecological Life Support System
MTP	Microtiterplate
NASA	National Aeronautics and Space Administration
NR	Nile Red
OD	Optical Density
OST	Outer Space Treaty
OTU	Operational Taxonomic Unit

PCA	Principal Component Analysis
PCR	Poly Chain Reaction
PGM	Platinum Group Metals
PHB	Polyhydroxybutyrate
PI	Propidium Iodide
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Scanning Electron Microscopy
SG	SYBR Green
SOD	Superoxide Dismutase
SSC	Side Scatter
TAS	Total Alkali Silica
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl- β -galactopyranoside

Table of Contents

Chapter 1 Introduction.....	1
1.1 Basalt: petrology, weathering and bacteria present.....	1
1.1.1 Basalt petrology	1
1.1.2 Lunar and Martian basalt.....	4
1.1.3 Abiotic weathering	8
1.1.4 Bacterial life on rocks	10
1.2 Microbe-mineral interactions	19
1.2.1 Bacterial microbe-mineral interactions on rocks	19
1.2.2 The space environment and application of microbe-mineral interactions	30
1.3 Choice of model organism and rock	38
Scope of the research.....	41
Chapter 2 Bacterial communities present on basalt deposits of different ages	45
2.1 Introduction.....	48
2.2 Material and methods	49
2.2.1 Sample sites and sampling.....	49
2.2.2 Culturing methods and identification of isolates.....	51
2.2.3 Composition analysis	52
2.2.4 DNA extraction	52
2.2.5 Illumina sequencing.....	52
2.2.6 Statistics.....	53
2.3 Results	54
2.3.1 Site parameters and lava flow composition	54
2.3.2 Basalt composition	54
2.3.3 Taxonomic distribution within each flow and comparison between de different flows and sites.....	55
2.3.4 Diversity and NMDS analysis between sites and comparison.....	58
2.3.5 Isolations.....	63
2.4 Discussion	64
2.5 Acknowledgements	70
Chapter 3 Growth in the presence of basalt	71
3.1 Introduction.....	74
3.2 Material and methods	75

3.2.1	Strain and media composition	75
3.2.2	Growth setup and analysis	75
3.2.3	Flow cytometry analysis	76
3.2.4	Elemental analysis	77
3.2.5	Basalt composition	77
3.2.6	Statistical analysis	77
3.3	Results	78
3.3.1	Growth kinetics.....	78
3.3.2	Effect of basalt on total cell fractions and physiology.....	80
3.3.3	Effect of basalt composition on element release.....	85
3.4	Discussion	86
3.5	Acknowledgements	89
Chapter 4	Survival in the presence of basalt	91
4.1	Introduction.....	94
4.2	Material and Methods	95
4.2.1	Basalt composition	95
4.2.2	Strain and media composition	95
4.2.3	Construction of PHB-negative mutant and complementation plasmid.....	96
4.2.4	Survival setup and analysis	97
4.2.5	Biofilm quantification and visualization setup and analysis	100
4.2.6	ICP-OES	101
4.2.7	Statistical analysis	101
4.3	Results	101
4.3.1	The effect of basalt on culturability, membrane permeability and energy status of <i>C. metallidurans</i> during 12-week storage.....	101
4.3.2	The effect of PHB production on culturability, membrane permeability and energy status.....	103
4.3.3	Biofilm formation on basalt	105
4.3.4	The effect of basalt on element release.....	108
4.4	Discussion	111
4.5	Acknowledgements	114
Chapter 5	Impact of space conditions on survival in the presence of basalt	115
5.1	Introduction.....	118
5.2	Material and Methods	119
5.2.1	Strain and media composition	119
5.2.2	Basalt composition	119

5.2.3	Flight setup	120
5.2.4	Post-flight analysis	121
5.2.5	ICP-OES	126
5.2.6	Statistical analysis	126
5.3	Results	126
5.3.1	The effect of space flight and basalt on cell survival and physiology	126
5.3.2	The effect of basalt and space flight on biofilm formation	129
5.3.3	The effect of basalt on element release	130
5.4	Discussion	134
5.5	Acknowledgements	137
Chapter 6	The use of flow cytometry to assess microbial viability...	139
6.1	Introduction	142
6.2	Viability	143
6.3	Dyes	145
6.3.1	Cell-permeant nucleic acid dyes	145
6.3.2	Functional dyes	149
6.4	Stain combinations	155
6.5	Stability	161
6.6	Conclusion	163
6.7	Acknowledgments	164
Chapter 7	General discussion and future perspectives	165
7.1	General research outcomes and positioning of this research	167
7.1.1	Positioning of this research	167
7.1.2	Main research outcomes	169
7.2	The impact of basalt age and composition on bacterial communities	171
7.3	Basalt as source of nutrients for bacterial growth	174
7.4	Basalt as source of nutrients for survival and substrate for biofilm formation ..	176
7.5	Future perspectives and applications	181
References	187
Appendix	219
Appendix 1:	Basalt compositions of all the basalt tested within this work	221
Appendix 2:	Material and methods section Chapter 6	222
Abstract	225

Samenvatting	229
CV	233
Dankwoord	239

Chapter 1 Introduction

Chapter 1 Introduction

In this PhD project, the interaction of bacteria with basalt rock was investigated in the framework of potential space applications. In this chapter, we summarized the composition of basalt, its chemistry, its presence on Mars and the Moon and possible use as substrate for extracting resources within in situ resource utilization (ISRU). Next, we reviewed possible interactions of bacteria with basalt and the mechanisms behind this rock-dwelling behaviour. Finally, we showed the application of microbe-mineral interactions in space.

1.1 Basalt: petrology, weathering and bacteria present

1.1.1 Basalt petrology

Basalt is the most common igneous rock type on the Earth's surface covering in total 60% of the land and the ocean floor (Winter, 2014). The basalt ocean floor forms the largest endolithic rock habitat on Earth, covering about 200,000 km² and expanding on average 2.6 km² per year (Meyerhoff et al., 1996). Basalt is formed through partial melts of mantle material, which will form oceanic divergent boundaries, hotspots, continental flood basalt and mantle plumes. Basalt formations are thus formed as new igneous material, in contrast to metamorphic rocks that are formed through the transformation of existing rock types. The composition of the formed basalt varies both in major and minor elements as well as trace and isotopic composition, serving as a probe for the mantle composition of the Earth (Kahl, 2015). In addition, basaltic rocks contain high concentrations of Cu, Mn, V and Zn compared to other igneous formations, as these elements can substitute for Fe, Al and Si during crystallisation of magma (Alloway, 2013). Most rudimentary, basalt can be classified according to the amount of silicates and the minerals formed during cooling of the original flow when it reaches the Earth's surface, in contrast to its plutonic formation into gabbro, described in the Bowen's reaction series (Figure 1.1 and 1.2). These series specify which minerals are formed at which temperatures and pressures while magma cools and predicts which minerals occur together.

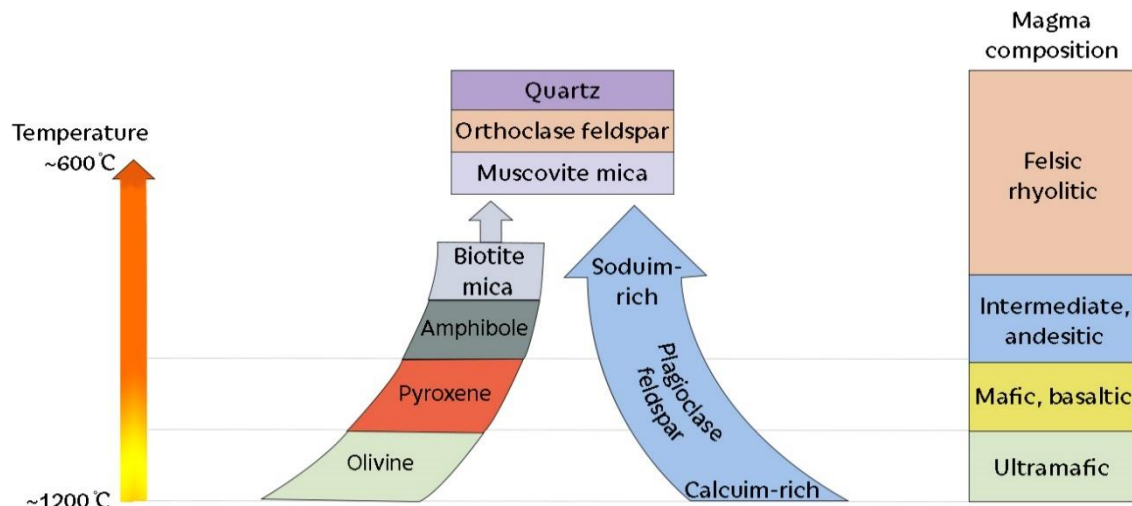


Figure 1.1: Bowen reactions diagram describing the influence of temperature and weathering on different formations

1.1.1.1 Basalt classification

Basalt is classified in the mafic category, containing 45 to 52 wt% of SiO_2 and 15 wt% of MgO , FeO , CaO and Al_2O_3 (Figure 1.2). Other elements are present at less than 0.1%. Major minerals that constitute basalt rocks are (a) olivine, of which Fe and Mg substitute freely for each other, within the continuous solid solution series, ranging from the pure iron Fe_2SiO_4 (fayalite) to the magnesium end member Mg_2SiO_4 (forsterite); (b) plagioclase, a mineral within the feldspar family which is, like olivine, part of the continuous solid solution series, where Ca and Na can substitute for another ranging from albite ($\text{NaAlSi}_3\text{O}_8$) to anorthite end members ($\text{CaAl}_2\text{Si}_2\text{O}_8$) (Fisk et al., 2006); (c) pyroxenes, which are rock-forming inosilicate minerals present in both igneous and metamorphic rocks, which are a single-chain silicates with the generic formula ($\text{M}_1\text{M}_2\text{T}_2\text{O}_6$). The T site is primarily occupied by Si or Al, and only M_2 can accommodate the larger Ca^{2+} ion. Fe^{2+} and Mg^{2+} cations can either occupy the M_1 or M_2 sites (Fisk et al., 2006); (d) inosilicates or chain silicates, which consist of interlocking chains of silicate tetrahedrals. At last it can also contain volcanic glass which is formed when magma rapidly cools down and which contains high amounts of silicates (Singha, 2012; Kahl, 2015).

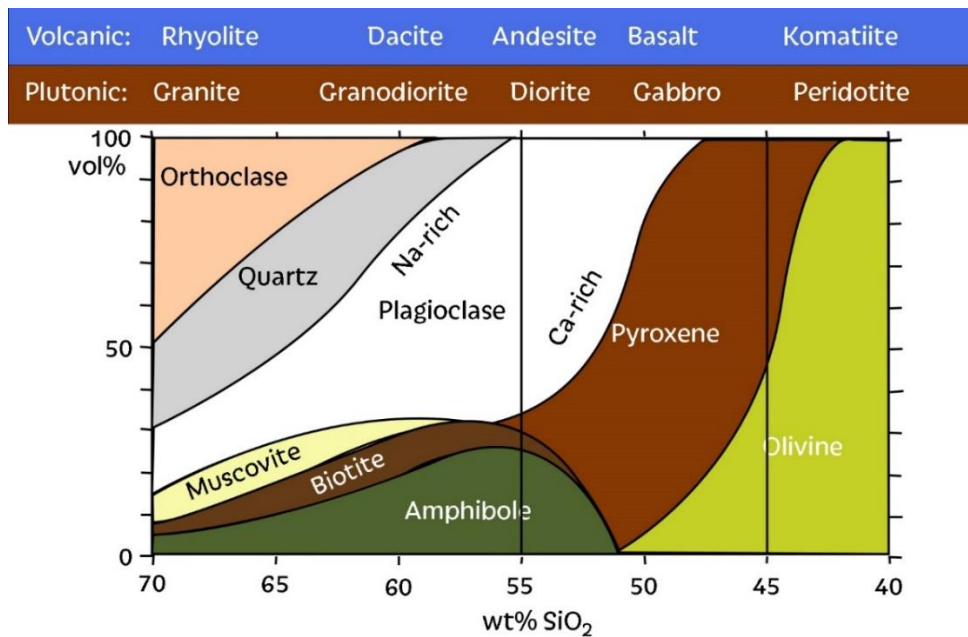


Figure 1.2: Classification of basalt according to its silica and mineral content formed through the Bowen series.

Classification can further be based on mineralogy by the use of the Total Alkali Silica (TAS) diagram (Figure 1.3) which was established by the International Union of Geological Sciences (IUGS) (Le Bas and Streckeisen, 1991; Le Bas et al., 1992). This classification categorizes basalts according to their silica (SiO₂) and alkali content (Na₂O and K₂O) (Le Bas and Streckeisen, 1991; Le Bas et al., 1992). This allows to subclassing 'intermediate' quartz and 'basic' olivine saturated basalts in "alkali" basalts and "tholeiitic" basalts. Alkali basalts are richer in alkali elements (Na₂O and K₂O) and poorer in CaO than tholeiites. Alkali basalt also contain less silica than their tholeiitic counterparts and tend to be higher in TiO, FeO and rare earth elements because these are the first elements to enter within a melt (Frost and Frost, 2013). The TAS diagram however is limited as not all basaltic rocks types can be classified. The TAS diagram can only be used for unaltered volcanic rocks that have not undergone alteration, crystal enrichment or contain low concentrations of silica (Le Bas et al., 1992; Hastie et al., 2009).

Therefore, other classification systems can be used such as the QAPF (Quartz, Feldspar, Plagioclase, Feldspathoid) crystal matrix where basalts can be divided according to their volcanic glass content. This is an important parameter, as some basaltic flows undergo rapid cooling when they come in contact with water or ice, resulting in the formation of homogenous glass and inhibiting crystal formation (Cockell et al., 2011).

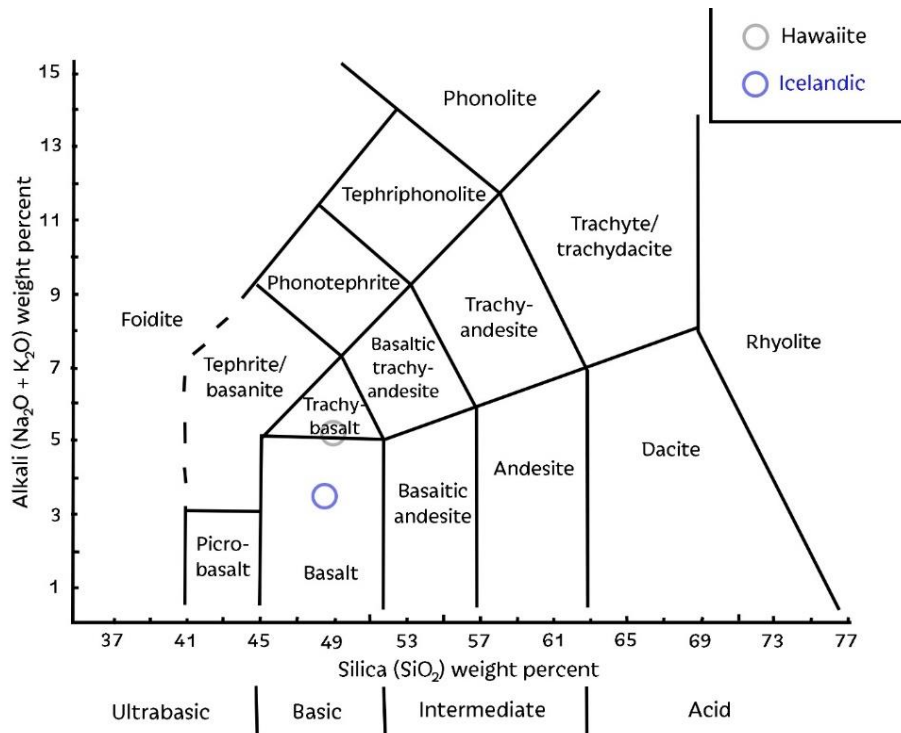


Figure 1.3: Visualization of the TAS diagram used to further classify basalt with the positions of Icelandic and Hawaiiite type formations of basalt. Adjusted from Stronck and Schmincke (2001).

1.1.2 Lunar and Martian basalt

In our solar system, basalt is a major component of the surfaces of the Moon, Mars, as well as Mercury, Venus, Io and some smaller asteroids and planetoids (Moskovitz et al., 2008; Wilson, 2009; McMahon et al., 2013). It is important to understand the composition of the regolith when considering it as possible and future substrate for extracting and mining resources for example to use in situ-resource utilization (ISRU) for future life support (Crawford, 2015).

First, the **lunar** regolith and surface can be categorized into four different geological groups (Table 1.1) based on their formation: (1) basaltic volcanic rocks, including lava flows and ash; (2) rocks from the lunar highlands; (3) complex polymix breccias that are formed by deformation of lunar material during shock impacts on the surface and (4) true lunar “soil” or regolith that covers the lunar surface (Heiken and Vaniman, 1990; Heiken et al., 1991). Hereby regolith is defined as “the layer of fragmental and unconsolidated rock material, of highly varied character, which covers most of the underlying bedrock and includes rock debris and ash of all kinds” (Bates and Jackson, 1987; McKay et al., 1991). The lunar surface consists of two main geological areas: the ancient, lunar highlands, and the darker, lunar maria or large impact basins. The lunar

highlands are composed of invariable Ca-rich anorthositic rocks ($\text{CaAl}_2\text{Si}_2\text{O}_8$) and small quantities of magnesium- and iron-bearing minerals such as pyroxene. Highland formations also contain KREEP rocks, which are highly enriched in potassium (K), rare earth elements (REE), and phosphorus (P) (Papike, 1998). The lunar maria are composed of basaltic lava flows and their composition is a combination of four plagioclase and pyroxene minerals: anorthite ($\text{CaAl}_2\text{Si}_2\text{O}_8$), orthopyroxene ($(\text{Mg,Fe})\text{SiO}_3$), clinopyroxene ($\text{Ca}(\text{Fe,Mg})\text{Si}_2\text{O}_6$), olivine ($(\text{Mg,Fe})_2\text{SiO}_4$) and the iron-oxide ilmenite (FeTiO_3). Maria basalts are thus richer in Mg, Fe and Ti, and relatively poorer in Ca and Al, which are the closest to terrestrial basalt, and more specifically midocean ridge basalts (MORB's) (Papike, 1998). The principal classification of lunar maria basalts is based on their Ti content: 'low Ti' if TiO_2 ranges from 1-6 wt% and 'high-Ti' if TiO_2 concentration is higher than 6 wt% (Neal and Taylor, 1992). The iron-oxide ilmenite could be a target for ISRU as these minerals contain volatiles for oxygen extraction. ISRU studies are usually performed with geochemical simulants for lunar regolith, i.e. simulant JSC-1 resembling the lunar mare samples (NASA, 2008; Stoesser et al., 2010) and simulant NULHT which composition is close to the anorthositic highlands (Schwandt et al., 2012). In general, lunar basalts contain more Fe and less Si and Al than terrestrial basalts as lunar lava formations cool faster. In addition, especially mare basalt, differs in many ways from terrestrial basalt and contains high concentrations of Ti, S, Cr and Fe while Mg, Al, Na and K concentrations are lower and lunar basalt is depleted of certain volatile elements such as K, Na, Rb, Pb, C and H but not all (Wolf et al., 1979). Lunar basalts are also depleted in siderophilic elements and elements that tend to concentrate in metallic iron (Ni, Co, Au, Ir, etc.) (Ryder and Schuraytz, 2001).

The surface of **Mars** contains two geological formations: the southern highlands, mostly consisting of basalt that subsequently weathered, and the northern lowlands, which contain more unaltered basaltic andesite (Wyatt and McSween, 2002; Taylor and McLennan, 2009). As Mars has undergone more extensive geological evolution compared to the Moon, including continued volcanism on a small-scale until the late Amazonian (Werner, 2009; McMahon et al., 2013), it has a wider variety of minerals and phases (e.g. oxides, glasses, sulfates, and clays; summarized Table 1.2) (Ehlmann et al., 2013).

Table 1.1: Summary of the presence of basalt compositions on the Moon

Igneous rocks	Volcanic mare basalt	Olivine basalt Low Ti basalt High Ti basalt Glass beads
	Highland rocks	KREEP anorthosite
Fragmental rocks	Breccias	Feldspathic breccia Regolith breccia Polymict breccia Impact melt breccia
	Regolith	Highland regolith Mare regolith

In terms of basalt, three different types of basaltic rock formations are present. The first is an older formation and consists of unaltered plagioclase and clinopyroxene rich basalt while the second basalt is higher in silica-rich phases, and has been suggested to be more andesitic. The third basalt formation consists of regolith-free rocks and which composition is closer to terrestrial andesitic basalts. The surfaces of each of these subtypes can be covered with secondary phases (silica glass, clays) and/or dust. In addition, other unique soil formations are present such as sandy-to-cloddy soil formations and sulphur-rich soils and rocks. These sulphur-containing soils and rocks contain high concentrations of S and Cl, which may suggest that low-pH acid-sulfate weathering was a major alteration pathway on Mars. Furthermore, in the Eagle crater, also jarosite, hematite, and Mg- and Ca-sulfates formations were found. Other formations may also indicate aqueous weathering processes. Jarosite is of importance as this is only formed under low pH by aqueous processes which may indicate that at one point during the geological evolution liquids were present at the surface of Mars. Furthermore, rocks at Columbia Hills show substantial leaching, leading to major Al-enrichment and the presence of montmorillonite and other clay smectite phases indicating again the presence of liquid and subsequent weathering. Lastly, almost all fine grained dust and soil particles on Mars are magnetic, because of the presence of a relatively unaltered igneous magnetite and maghemite minerals suggesting that

aqueous alteration must have been present but not sufficient to alter a significant fraction of Mars' regolith (Vaniman et al., 1991; McLennan, 2003; McSween et al., 2004; Bibring et al., 2006; Carr and Head, 2010).

Table 1.2: Summarizing of mineral phases found on the Martian surface (based on Bell (2008)).

Mineral or Material found	Results
Carbonates: e.g., Calcite (CaCO_3); Siderite (FeCO_3); Magnesite (MgCO_3); Scapolite ($\text{Ca}_4[\text{Al}_6\text{Si}_6\text{O}_{24}]\text{CO}_3$); Artinite ($\text{Mg}_2\text{CO}_3(\text{OH})_2 \cdot 3\text{H}_2\text{O}$); Hydromagnesite ($\text{Mg}_5(\text{CO}_3)_4(\text{OH})_{24}$)	Simple carbonate phases not detected in significant abundances; minor amounts possible in dust and in some spectra, hydrated forms lack strong carbonate features. Telescopic evidence for surface bicarbonate anions, but specific identification uncertain
Sulfates: Kieserite ($\text{MgSO}_4 \cdot \text{H}_2\text{O}$); Gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$); "Polyhydrated sulfate" $[(\text{Fe}, \text{Mg}, \text{Ca}) \cdot n\text{H}_2\text{O}]$; Anhydrite (CaSO_4); Jarosite $[(\text{K}, \text{Na})\text{Fe}_3(\text{SO}_4)_2(\text{OH})_6]$	Telescopic evidence for SO_4^{2-} anionic group absorptions but specific identification of any of these phases was uncertain. Specific sulfate identifications ultimately came from later MER and OMEGA measurements
Fe-oxides: "Red" hematite: ($\alpha\text{-Fe}_2\text{O}_3$) 'Nanophase ferric oxides; Goethite: ($\alpha\text{-FeOOH}$) Lepidocrocite ($\gamma\text{-FeOOH}$); Ferrihydrite ($\text{Fe}_2\text{O}_3 \cdot n\text{H}_2\text{O}$); Schwertmannite ($\text{Fe}_8\text{O}_8(\text{OH})_6\text{SO}_4$); Magnetite (Fe_2O_4): ; Maghemite (Fe_2O_3)	Identified in telescopic and ISM spectra from crystalline ferric bands near 0.65 and 0.9 nm Inferred from telescopic, spectral analog studies as the strongly pigmenting material responsible for the visible color of Mars, but lacking crystalline ferric absorptions in the near-IR. Inferred from analysis of some Mariner IRS and Viking orbiter multispectral images; definitive identification uncertain. Inferred from analysis of some telescopic studies from visible color and ferric absorption band near 0.9 nm, but definitive identification uncertain
Phyllosilicates: Montmorillonite (Al, Mg smectite); Nontronite (Fe smectite); Greenalite, Chamosite, Cronstedtite (Fe serpentine)	Weak bands in the 2.0 to 2.5 nm range detected in some telescopic and ISM spectra, but specific layer silicate identification equivocal.
Pyroxene: Low-Ca (LCP): Enstatite ($\text{Mg}_2\text{Si}_2\text{O}_6$); Pigeonite ($[\text{Mg}, \text{Fe}, \text{Ca}]\text{Si}_2\text{O}_6$); High-Ca (HCP): Diopside ($[\text{Ca}, \text{Mg}, \text{Fe}]\text{Si}_2\text{O}_6$); Augite ($[\text{Ca}, \text{Na}, \text{Mg}, \text{Fe}](\text{Si}, \text{Al})_2\text{O}_6$)	Identified in telescopic and ISM spectra from distinctive 1- and 2-nm absorption features. Both LCP and HCP detected and mapped based on band center variations tied to laboratory pyroxene spectral studies.
Olivine: Forsterite (Mg_2SiO_4); Fayalite (Fe_2SiO_4)	Inferred from analysis of some telescopic studies from weak absorption near 1.2 to 1.5 nm, but definitive identification uncertain.
"Hydrated minerals"	Detected telescopically and by IRS at the 1–3 wt% level based on ubiquitous presence of 3-nm

	band, but specific hydrated mineral identification uncertain.
CO ₂ ice	Detected in both seasonal polar caps.
H ₂ O ice	Detected in north residual polar cap and as an enhancement in the edge of the seasonal cap.

1.1.3 Abiotic weathering

Exposure of minerals to rainwater, temperature changes and oxidative conditions results in abiotic or chemical weathering reactions, including dissolution and crystallization of minerals (Uroz et al., 2009). This chemical weathering of crustal rocks is one of the principal processes controlling the geochemical cycle of elements at the Earth's surface. Chemical weathering consumes atmospheric carbon dioxide and leaches metals from the rocks, while releasing oxygen and water (Garrels and Mackenzie, 1971). Basalts are among the more easily weathered than other crystalline silicate rocks. It has been shown by Louvat and Allègre (1997) that basalt weathering acts as a major atmospheric CO₂ sink (Louvat and Allègre, 1997; Dupré et al., 2003). Dessert et al. (2003) therefore estimated the CO₂ flux consumed by chemical weathering of basalts to be about 4.08×10^{12} mol/year with the fluxes of the islands of Indonesia and regions of central America contributing the major part (40%) of this flux. The global flux of CO₂ consumed by chemical weathering of basalts represents 30% to 35% of the total flux derived from continental silicate weathering (Gaillardet et al., 1997). In addition, it appears that volcanic activity not only acts as a major atmospheric CO₂ source, but also creates strong CO₂ sinks that impact the geochemical and climate evolution of the Earth (Dupré et al., 2003).

Among all rock types, basalts are particularly sensitive to chemical weathering and the products of basalt weathering varies according to the geochemical environment (Staudigel and Hart, 1983; Daux et al., 1994; Berner and Berner, 2012). It has also been shown that parameters such as vegetation, organic acids (Gislason and Oelkers, 2003) and the age as well as the composition of rock basalt substrates (Kennedy et al., 1998) can influence the chemical weathering of silicates. Many of the earlier studies of basalt weathering have been summarized by Loughnan (1969) and as basalt is rich in reduced element species, it is therefore susceptible to redox gradients, which are i.e. present in seawater, and which impact speciation of the different elements present (Edwards et al., 2005). It was seen that basalt dissolution is incongruent as different

primary minerals do not weather at the same rate and as a result leached elements are heterogeneously partitioned between the solution and formed secondary minerals (Aiuppa et al., 2000). Colman's study established a mineral susceptibility sequence for basalt in which glass weathers first followed by olivine, pyroxene, amphibole, plagioclase and lastly, if present, K-rich feldspar respectively (Colman, 1982). Volcanic glass is the most susceptible component of basaltic rocks and it incongruently alters to "palagonite", which is a mixture of secondary precipitation reactions (Stroncik and Schmincke, 2001; Stolz and Oremland, 2011). Almost as susceptible is olivine, which may alter to saponite or nontronite or after loss of magnesium and oxidation of iron, to hematite, maghemite, or goethite (Craig and Loughnan, 1964). Pyroxenes weather to trioctahedral smectite, whereas plagioclase alters to dioctahedral smectite and K-rich feldspar weathers to kaolinite or halloysite. Ultimately, all these minerals weather to a mixture of iron oxide-hydroxides and clay minerals rich soils called andosols, which form an important part of the terrestrial volcanic environment. Andosols are present, as acidic pH soils, around geothermal pools, hot and cold desert soils (Óskarsson et al., 2004). Andosols generally possess a good soil structure, water retention, and porosity; yet these soils are prone to erosion (McDaniel et al., 2012). They are generally high in Fe and Al. These metals, in addition to high concentrations of humic acids, give andosols characteristic high phosphate (P) retention potential (Lukito et al., 1998). This retention of biologically available P critically impedes the potential for microbial and floral growth on younger andosols (Kimble et al., 2000).

In terms of single elements released from basalt (and also other rocks) at neutral pH and within atmospheric temperatures ($T < 25^{\circ}\text{C}$), Ca, Mg, K, Na, Rb and Sr generally show a rapid, early loss that can be correlated with the alteration of volcanic glass to smectite and weathering of plagioclase. This palagonitization of volcanic glass is a continuous process of glass dissolution, palagonite formation, and palagonite evolution, which can be subdivided into two different reaction stages. The first stage is characterized by congruent dissolution of glass and basalt losing Si, Al, Mg, Ca, Na, and K, while there is an enrichment of Ti, Fe and H_2O . The second stage consists of palagonite reacting with the released elements and fluid leading to crystallization of smectite. Hereby, Si, Al, Mg, and K are taken from the solution while Ti and H_2O are lost from the solution. The degree and direction of the element mobility during palagonitization varies with palagonite age (Stroncik and Schmincke, 2001). Other

elements such as Mn, P, Cu, and Zn appear to be more slowly and uniformly leached; Ti, V, Cr, Ni, Zr, and Nb are essentially immobile while rare earth elements and Ba are initially lost (Eggerton et al., 1987). In addition, this also impacts pH and during the second phase of incongruent dissolution, the pH will increase through proton-exchange at the surface. This non-stoichiometric release of elements of the initial reaction also leads to the formation of a basalt surface layer depleted of Ca, Mg and Na (Staudigel and Hart, 1983; Daux et al., 1994).

1.1.4 Bacterial life on rocks

Regardless of their origin and location, all mineral and rock environments can constitute habitats which can be colonized by microorganisms (Table 1.3). This microbial colonization of minerals seems to be an ancient strategy (Furnes et al., 2007; Ivarsson et al., 2012) and life on Earth probably originated within a mineral habitat by successfully colonizing and interacting with a diverse array of rocks and minerals (Ferris, 2005; Uroz et al., 2015).

Rocks can be a beneficial environment for microorganisms by physically supporting the attachment of microorganisms within cracks, fissures, cavities and pores in the rocks, providing shelter from harsh conditions and serving as nutritive reserves (Barker and Banfield, 1998). This 'supporting' effect of rock composition constituting specific microbial habitats which control microbial community establishment led Uroz et al. (2015) to propose that bacteria inhabiting these rock environments are part of the 'mineralosphere'. This colonization is dependent on the physical attributes of minerals as well as porosity and size of the minerals allowing preferential accumulation along the edges of mineral particles (Barker et al., 1998; Bennett et al., 2001). Minerals can be considered as physicochemical interfaces that release elements, absorb compounds on their surface, as well as form precipitates. Many of the elements (e.g., Fe, Mn, Mg, P, Ca, and Na) entrapped in minerals are physiologically required by bacteria as electron donors, terminal electron acceptors, cofactors or nutrients (Bennett et al., 2001; Hutchens et al., 2010; Uroz et al., 2015).

Table 1.3: Summary of the different bacterial groups found within different rock dwelling environments

Endolith in the rock		
Dominant	Desert	Non-desert
Mostly cyanobacteria	<i>Chloroflexi</i> , fungi and mosses	Filamentous and unicellular types of cyanobacteria, such as <i>Leptolyngbya</i> , <i>Nostoc</i> , and <i>Synechocystis</i>
Epilithic on the rock		
Dominant	Biofilm	Non-biofilm
Epilithic lichens and algae	Cyanobacteria related to <i>Xenococcus</i> , <i>Myxosarcina</i> , <i>Chroococcidiopsis</i> , <i>Blennothrix</i> , <i>Pseudanabaenaceae</i> , <i>Calothrix</i> , <i>Entophysalis</i> and <i>Lyngbya</i> additional <i>Bacteroidetes</i> , <i>Actinobacteria</i> and <i>Proteobacteria</i>	<i>Chroococcidiopsis</i>
Cryptoendolithic internal cavities and interstices of rocks		
Dominant	Biofilm	Non-biofilm
lichens, cyanobacteria <i>Phormidium</i> and <i>Plectonema</i>	α - <i>Proteobacteria</i> and <i>Thermus-Deinococcus</i>	<i>Geobacteriaceae</i>
Basalt		
Dominant	Marine	Continental
<i>Actinobacteria</i> , <i>Proteobacteria</i> and <i>Chloroflexi</i>	Heterotrophs, chemolithotrophs Epsilonproteobacteria closely related to sulfur-oxidisers. Iron-reducing organisms <i>Firmicutes</i> and <i>Crenarchaeota</i>	Cyanobacteria related to <i>Anabaena</i> and <i>Nostoc</i> <i>Acidobacteria</i> , <i>Alphaproteobacteria</i> capable of CO ₂ oxidation

Analysis of different minerals, such as muscovite, plagioclase, K-feldspar and quartz, extracted *in situ* from various rocks, revealed that the bacterial community structure varied in relation to the chemical composition of the minerals, with some phylotypes present only in certain minerals. Colonization factors depended on local niche conditions and elements, such as sodium and silica, which were identified as key structuring elements (Rogers and Bennett, 2004; Gleeson et al., 2006). For example, Mitchell et al. (2013) placed small crystals of pyrite, hematite, magnetite, olivine, calcite and quartz in the glacial meltwater stream at Robertson Glacier (Canada) and showed that both structure and composition of the colonizing bacterial communities as well as

the amount of biomass were affected by the mineral geochemistry, in particular the iron content. They hypothesized that mineral surfaces were preferentially colonized when electron donor or acceptors (pyrite, hematite, and magnetite) were present. Here, iron is often limited because of the formation of insoluble iron oxides under aerobic conditions. Microbes can recognize these iron-containing minerals and accelerate the release of iron from these minerals (Ehrlich, 1996;Kalinowski et al., 2000;Mauck and Roberts, 2007;Dong, 2010). In addition, microbes preferentially interact and colonize surfaces that possess available phosphorus, such as apatite, olivine and feldspars because of the low availability of phosphorous from other phosphorous-containing minerals (Rogers and Bennett, 2004;Mailloux et al., 2009).

Several studies also showed that microbial colonization on mineral surfaces was affected by the temperature, pH, light, UV irradiation as well as oxygen, water, carbon availability and ionic strength and element availability of the minerals (McKay and Friedmann, 1985;Hughes and Lawley, 2003;Chan et al., 2012;Štyriaková et al., 2012). Also phages can impact the abundance and structure of such a microbial rock dwelling community, but this has not yet been fully documented (Olsson-Francis et al., 2010a;Antony et al., 2012;Olsson-Francis et al., 2015). For water availability, rock dwelling bacteria produce intracellular retention molecules such as trehalose and sucrose which have liquid retaining characteristics (Friedmann et al., 1993), enabling them to resist repeated cycles of desiccation within the rock pore space environment (Wynn-Williams et al., 2002). Endolithic microorganisms found in the Antarctic Dry Valleys and the Canadian High Arctic also produce extracellular polysaccharides to avoid desiccation and minimize the damaging effects of freeze–thaw cycles (Omelon, 2008). Not only water and desiccation conditions but also UV radiation could arrest the growth and proliferation of rock dwelling or endolithic communities in extremely dry deserts (Quesada et al., 1999). These endoliths produce UV protecting compounds such as scytonemin and carotenoids which could aid in the protection against radiation (Cockell et al., 2003). For nutrient availability, atmospheric deposition from precipitation and dust are likely to contribute to the available nutrient pool (Friedmann et al., 1993).

1.1.4.1 Rockdwellers

The dominant bacterial species in rock dwelling environments, also called endoliths are phototrophs, such as cyanobacteria, are either free living or endosymbionts in lichens (Friedmann, 1980). Members of the cyanobacterial phyla as well as *Chloroflexi*, fungi and mosses are widely reported to be the dominant hypoliths present in arid environments such as hot and cold deserts, living underneath rocks (Cockell and Stokes, 2004; Warren-Rhodes et al., 2006; Antony et al., 2012). Conversely, in non-desert environments such as dolomitic rocks in Switzerland and travertine deposits in Yellowstone National Park, the endolithic communities, present inside the rock, are more diverse and include both filamentous and unicellular types of cyanobacteria, such as *Leptolyngbya*, *Nostoc*, and *Synechocystis*. In addition thermophilic autotrophic bacteria such as *Aquificales*, green non sulfur bacteria as well as β -Proteobacteria were found present in hot springs (Fouke et al., 2003; Olsson-Francis et al., 2010a; McMahon et al., 2013).

Epilithic microorganisms, colonizing the surface of rocks, include both unicellular and filamentous forms, for example, *Lyngbya*-related species and *Chroococcidiopsis* (Bruno et al., 2006). Epilithic lichens and algae have also been found within Antarctic rocks (Pizarro et al., 1996). Biofilms formed by epilithic organisms on the surface of rocks contain thick-sheathed cyanobacteria related to *Xenococcus*, *Myxosarcina* and *Chroococcidiopsis* spp. in addition to members from the divisions *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (Narváez-Zapata et al., 2005). Mats and biofilms on the surface of the rocks of the Great Barrier Reef, Australia, showed a high diversity of cyanobacterial phylotypes related to the genera *Blennothrix*, *Pseudanabanaceae*, *Chroococcidiopsis*, *Calothrix*, *Entophysalis* and *Lyngbya* (Díez et al., 2007). The cryptoendolithic community, present within the internal cavities and interstices of sandstone rocks in the McMurdo Dry Valleys of Antarctica was found to be dominated by lichens, cyanobacteria belonging to the genera *Phormidium* and *Plectonema*, and other bacteria belonging to the α -*Proteobacteria* and *Thermus-Deinococcus* classes (José et al., 2003). Cryptoendolithic biofilms on porous sandstones from Colorado Plateau, USA, were dominated by cyanobacteria and *Geobacteriaceae* (Kurtz et al., 2005; Antony et al., 2012).

Chroococcidiopsis, which have been found in many of these studies, has also been proposed as a suitable 'pioneer' organism for terraforming planets such as Mars (Friedmann et al., 1993).

1.1.4.2 Rockdwellers on oceanic and terrestrial basalt

As ocean crust could be an analogue to that formed during Early Earth, understanding the bio-alteration of basaltic glass is important for understanding how life developed on Early Earth (Staudigel et al., 2008; Schulz et al., 2013). The seafloor basalt-water-microbial interface has been extensively studied, proving that microorganism colonizing basalt surfaces play a part in the process of biogeochemical cycling of elements within the biosphere and release into ocean water (Cockell et al., 2011). Basaltic glass contains a large portion of reduced transition metals that may serve as energy source in submarine and terrestrial volcanic settings such as iron and manganese. Subsequent microbial-mediated dissolution of these elements releases nutrients that could drive subsequent colonization and carbon fixation (Bailey et al., 2009; Kelly et al., 2010; Cockell et al., 2011; Olsson-Francis et al., 2015).

As mentioned, the best studied igneous rock formations are **oceanic basalts** and communities dwelling with the rock-seawater-microbial interface have been extensively studied. Bacteria present the majority (90%) of the prokaryotic population, Archaea constitute a minority and their ecological role is not well known (Lysnes et al., 2004). It was estimated that up to sixty percent of all bacteria and Archaea live below the Earth's surface and as many as 6×10^{30} cells are present below the Earth's surface (Whitman et al., 1998). Microorganisms in the deep subsurface have been discovered at a depth of 1.5 km in oceanic crust and up to 4 km in terrestrial crust (Konhauser et al., 2007; Mason et al., 2010). Below these depths, the heat radiating from the Earth's mantle approaches the upper temperature limit of life i.e. 120°C (Konhauser et al., 2007). Lysnes et al. (2004) reported that basalt of varying ages in the Arctic region harbors different phyla, for instance *Actinobacteria* were associated with older basalt formations but were absent in freshly erupted material. Therefore, the abundance and presence of different phyla may correspond to differences that impact available carbon and electron resources which allow microbial metabolism. In addition, porosity affects the extent to which rocks can be colonized (Lysnes et al., 2004; Mason et al., 2009). For the microbial community present within the basaltic glasses of the Knipovich Ridge, Thorseth et al. (2001) identified heterotrophs and some chemolithotrophs

epsilonproteobacteria closely related to sulfur-oxidisers. Iron-reducing organisms were cultured from Arctic Ridge seafloor basaltic glasses by Lysnes et al. (2004) as well as isolates belonging to the *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria* and *Crenarchaeota*, implying iron-cycling within seafloor basalts (Thorseth et al., 2001). Phylogenetic analysis of seafloor basaltic glasses around Hawaii showed high diversity, which is hypothesized to be linked to the chemolithotrophic use of basaltic glass alteration products (Santelli et al., 2009; Henri et al., 2015).

Although the potential geochemical nutrient and energy availability from deep-ocean and terrestrial basaltic rocks are similar because of similar basalt formation and composition, the deep-ocean environment is very different from the **terrestrial** one. The latter, in contrast to the deep ocean, is exposed to freshwater in the form of snowmelt, acidic rainwater, large temperature fluctuations and periodic desiccation. Results from Cockell et al. (2013) on Icelandic basalt suggest that the majority of the active microbial population within volcanic rocks must depend on carbon input available as precipitated substrate, in the form of other dead or decaying organisms, or carbon produced by phototrophs and other autotrophs (Cockell et al., 2009). Early colonization of basalt could also depend on exogenous precipitation of carbon and energy sources such as trace gases (CO, H₂, CH₄) (Gomez-Alvarez et al., 2007). In addition, most research on the initial colonization of terrestrial rocks focused on the role of lichens, while research on bacteria and their role in volcanic rock weathering was only recently elucidated with diversity studies showing that *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* were found to be the dominant phyla (Gomez-Alvarez et al., 2007; Cockell et al., 2009; Kelly et al., 2010; Kelly et al., 2011; Fujimura et al., 2012; Kelly et al., 2014). Some differences are seen depending on mineralogical type and origin, time of eruption of the basalt flows (Cockell et al., 2009; Kelly et al., 2011). For Icelandic lava flows, through culture based and microscopy techniques, mostly cyanobacteria such as *Anabaena* and *Nostoc* were found on Heimaey island (Iceland) 18 months after an eruption. Icelandic volcanic rocks, formed around the Eyjafjallajökull formations, revealed the presence of *Acidobacteria*, *Actinobacteria* and *Proteobacteria*. For Hawaii deposits, communities were different from the Icelandic population, and were dominated by not only *Cyanobacteria* and *Acidobacteria* but also *Alphaproteobacteria* and were shown to be capable of CO₂ oxidation (Kelly et al., 2010; Kelly et al., 2014). In Hawaiian deposits formed in 1959, *Acidobacteria*,

Alphaproteobacteria were found while containing a large percentage of unclassified OTUs. Gomez-Alvarez et al. (2007) only found *Actinobacteria* and *Acidobacteria* in different lava flows on Hawaii formed back to 1700, and although it is not clear to what extent specific bacterial phyla are endemic to this environment, *Actinobacteria* are suggested as having a key role in basalt colonization and weathering (Cockell et al., 2013). From other more weathered basalt environments, such as forested lava formations, *Proteobacteria* dominated while also containing a high number of unclassified *Chloroflexi* sequences (Cockell et al., 2009). Communities present on unvegetated flows formed around the Mount St. Helen volcano, Washington, both *Alpha* and *Betaproteobacteria* were found as well as *Actinobacteria*, seventeen years after the eruption (Ibekwe et al., 2007).

Within andosols, the end product of basalt weathering, it has been shown that microorganisms were key to the release of organic and inorganic P, making it available for plant adsorption in young andosols (Richardson, 2001; Athanase et al., 2013). Nüsslein and Tiedje (1998) investigated a 200-year-old andosol in Hawaii and found that it was dominated by *Pseudomonas*, *Rhizobium*-*Agrobacterium*, and *Rhodospirillum* species containing a highly diverse population.

1.1.4.3 Moon and Mars environment and potential past life

By investigating Earth's habitability trajectories, knowledge could be gained with which to assess the habitability of diverse rocky extrasolar planets (Fridlund and Kaltenegger, 2008). 'Habitability' is a conservative term, limited by our knowledge of the range of possible lifeforms, their environmental and metabolic capabilities, and their energy requirements (Fridlund and Kaltenegger, 2008; Cockell, 2014).

As the **Moon** has no atmosphere or magnetic field, did not undergo extensive geological evolution and virtually no liquid water was or is available, either on the surface or subsurface, the presence of past and present life is nearly excluded (Table 1.4). However, recent research may show that the Moon's surface may contain some forms of liquid water (Anand et al., 2012), formed around craters near the lunar poles where 'cold traps' are formed due to permanent shadow (Ichimura et al., 2012). Analysis of lunar samples and remote-sensing data indicated that the lunar surface is however highly depleted in water compared to the Earth (Haskin and Warren, 1991; Crawford, 2015). In addition, liquid water could be formed through impact events

or the interaction of He/H particle lunar radiation winds interacting with the surface and forming liquid pools around the lunar poles (Papike et al., 1982).

Table 1.4 : Summarizing table comparing different parameters of temperature, pressure, radiation levels for the Earth, the Moon, Mars and asteroids (according to McKay (1991), Adams (2007), Cockell (2010b) and Hassler (2014)).

Conditions	Earth	Moon	Mars
Temperature (°C)	-89 to +50 Average 15°C	-223 to 123 Average -76°C	-140 to +20 Average -60°C
Pressure (mbar)	1013	3×10^{-12}	6
UV radiation	smaller than 290 nm (ozone)	unfiltered	smaller than 200 nm (carbonate column)
Cosmic ionizing radiation (mGy/day)	0,05	0,3-1	0,1-0,3
Gravity (g)	1	0,17	0,38
Atmosphere composition	78% N ₂ , 21% O ₂ 0,03% CO ₂	/	95% CO ₂ , 2,7% N ₂ , 1,6% Ar, 0,13% O ₂

Volatiles of helium and hydrogen are present on, or around, the lunar surface (Hodges et al., 1974) and the Lunar Crater Observation and Sensing Satellite provided direct evidence of its presence by crashing an empty rocket stage into the lunar surface of the south pole (Schultz and Wrobel, 2012). Next, lunar regolith also contains substantial quantities of all the main elements required for sustaining life (C, H, N, O, S, K, Ca, Mn), except phosphorus. Phosphorus is, however, present in KREEP rocks (Warren and Wasson, 1979). In addition, other trace elements such as Co, I, Mo, Va, Se and Cu can also be found on the Moon. In addition, platinum group metals (PGM's), Fe, Ti, Al, Si and rare earth elements are also found in the regolith and sufficient extraction methods could be developed in order to extract these elements (Anand et al., 2012; Crawford, 2015). The Lunar regolith, like Mars regolith, contains sources which could support future lunar outposts and regolith could be abiotically processed to be used as radiation shielding and 3D printing of the necessary habitats. Next, regolith could also be used to produce biofuels such as hydrogen, ethane, methane, kerosene, methanol and ethylene (Kading and Straub, 2015; Lim and Anand, 2015). These products could be further processed and used as rocket propellants. Oxygen could be produced from solid TiO₂ (Chen et al., 2000a). Regolith in itself could also be

processed such that it can serve as a matrix to stabilize loose regolith, adhesives, or lubricants (Montague et al., 2012).

Present day conditions on **Mars** with its low temperate, thin atmosphere as well as the absence of a magnetic field and therefore high surface exposure to UV and solar radiation make it unsuitable for life (Table 1.4). The shallow subsurface below Mars' surface, where life will be sheltered from these extremes, forming a warmer and possibly wetter environment could be more conducive for life (Aubrey et al., 2006; Dartnell et al., 2007; Popa et al., 2012). The subsurface is also protected from oxidants likely to destroy biomolecules (Benner et al., 2000). Lava tubes found on Mars could be an ideal habitat for life as conditions at the surface within these lava tubes could allow the presence of liquid water as well as Fe(II)-bearing minerals, which could be a source of energy while life would still be protected from radiation (Newman, 2010). In addition, even if life is not present anymore, these lava tubes could still allow for the preservation of biosignatures such as microbial mats, mineral bio-weathering features, unaltered biological sediments, biologically produced and trapped volatiles and organics, and the build-up of gases from biotic rock weathering formations (Northup et al., 2011). Therefore, the Martian shallow subsurface and lava caves have been also proposed as astrobiological relevant sites to look for signatures of life on Mars (Boston, 2010; Northup et al., 2011).

Thus on Mars, potentially, rocks and their reactions with the water possibly present in the subsurface or in lava tubes could provide a broad palette of metabolic substrates and essential nutrients to support life (Boston et al., 1992; Fisk et al., 2006; McMahon et al., 2013). Possible resources on Mars to support life have been summarized in detail by Cockell (2014) and Verseux et al. (2016a). In fact, most elements needed to support life have been detected in the Martian soils and rocks, such as the basic building blocks (C, H, O, N, P, S) and macro and minor elements (Mg, Fe, Ca, Na, K, Mn, Cr, Ni, Mo, Cu, Zn...). Many organisms need organic compounds as carbon and energy sources, and their state and availability on Mars remains poorly known but is likely low Ming et al. (2014). The main limitation comes from the abilities of microorganisms to use them under the form they are encountered on the surface of Mars. There is gaseous carbon and nitrogen in the atmosphere and fixed nitrogen compounds have also been detected (Ming et al., 2014). Water can be present as ice at the north and south polar ice cap, in the subsurface, as mineral hydration and as vapor in the atmosphere

(Tokano and Bish, 2005). Liquid water was probably present for a part of its geological history and may still exist at the rock-ice interface, in brines, as a results of impact events or as groundwater (Carr and Head, 2010; Clifford et al., 2010; Martín-Torres et al., 2015).

Diverse **biomarkers** could be used to understand present and past biological activities rocks. Most biological molecules cannot survive diagenesis and metamorphism, but lipid biomarkers are an exception: they can be preserved in sedimentary rocks and used to infer past microbial communities (Olcott et al., 2005). For ancient and highly altered rocks, however, geochemical biomarkers, i.e. biogenic minerals as products of the microbial activity, may be more robust. Within basalt, glass alteration structures have been most studied, i.e. stalks produced by iron-oxidizing bacteria and tubular textures in basaltic glass (Staudigel et al., 2008). But only a few minerals are known to be exclusively biogenic, including rosickýite and hazenite ($\text{KNaMg}_2(\text{PO}_4)_2 \cdot 14\text{H}_2\text{O}$) (Hazen et al., 2008). A method to test the biogenic origin of these formations is by using spectroscopic characterization after thermal treatment. Untreated biogenic carbonates (aragonite and calcite) show the same characteristics as abiotic carbonates in infrared spectra, but after heating, the biogenic spectra are different (Orofino et al., 2010). Compound-specific stable isotope fractionation of minerals is another powerful tool for understanding the role of microbial metabolisms in the formation of the minerals, as biogenic processes lead to variations in isotopes fractions, that are preserved in the rock record (Sharp, 2007). In addition to the research on conventional isotopes, such as C, N, and S, recent studies have focused on non-traditional isotopes, such as Mg, Ca, Cr, Mo, Fe, Cu, Zn, Cd, and Hg (Bullen and Eisenhauer, 2009).

Even though basalt is the dominant rock type in Martian regolith, the Martian regolith still needs a (micro)biological treatment before it is suited to support the growth of plants because of their poor water-holding properties due to its low organic carbon contents (Maggi and Pallud, 2010; Cockell, 2011).

1.2 Microbe-mineral interactions

1.2.1 Bacterial microbe-mineral interactions on rocks

The origin and evolution of life is thought to have been catalyzed by minerals, and microbe-mineral interactions have transformed Earth's surface mineralogy. Sulfur

metabolizing bacteria were responsible for pyrite precipitation and sulfur isotope fractionation (Wacey et al., 2011). *Cyanobacteria* formed carbonate precipitations as early as 3.5 Ga ago (Altermann et al., 2006). Not only bacteria but also lichens have been shown and widely studied for their rock weathering in a wide range of habitats (Dong, 2010; Gadd, 2010; Bjelland et al., 2011). Microbe-mineral interactions are important in bioweathering, within different artificial and natural ecosystems, soil fertility, plant growth promotion, bioremediation and bioremediation of inorganic pollutants (Figure 1.4) (Mapelli et al., 2012).

1.2.1.1 Mechanisms of microbe-mineral interactions

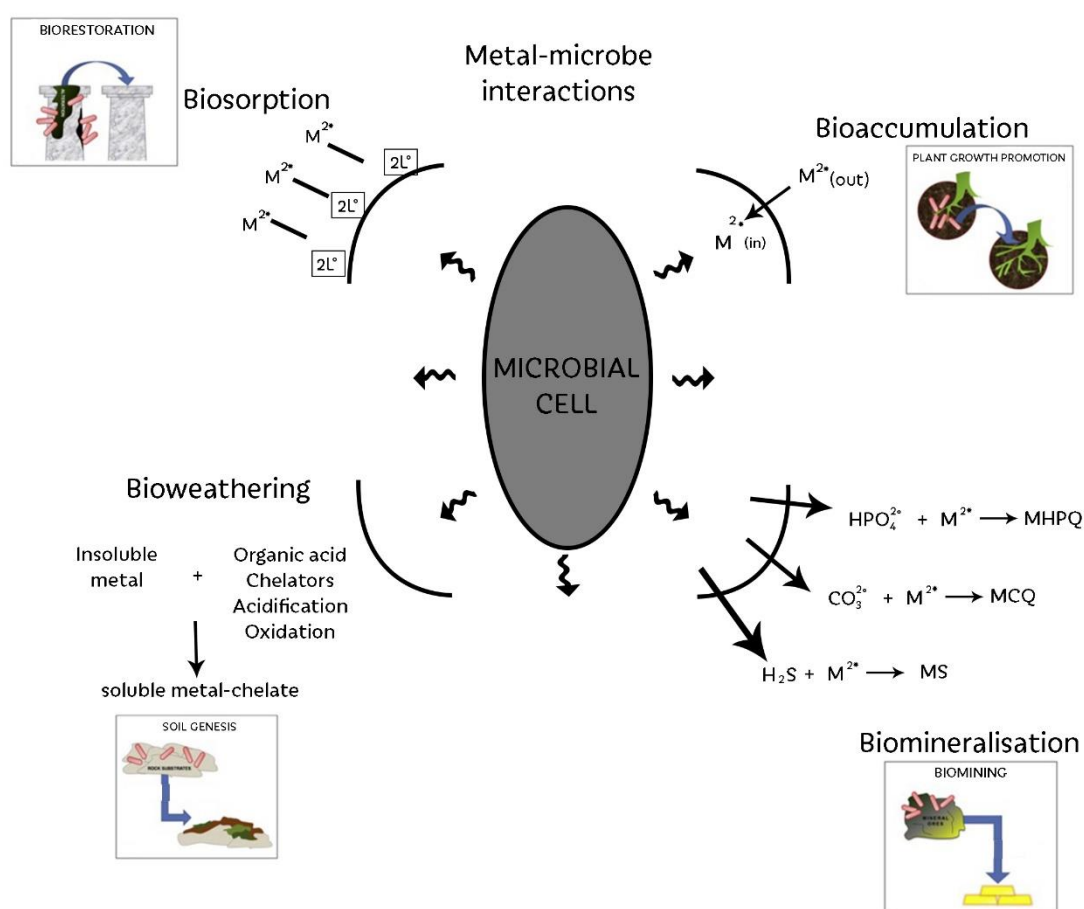


Figure 1.4: Overview of microbe-mineral interactions, their potential and applications. Adapted from Tabak et al. (2005) and Mapelli et al. (2011).

Microbe–basalt interactions can directly accelerate elemental release and **bioweathering** from geologic materials, through the acquisition of limiting nutrients required for biomass synthesis, influencing saturation state, oxidation and reduction of certain elements (Bennett et al., 2001; Welch et al., 2002; Gadd, 2010). This can also happen indirectly via release of organic acids (Welch and Ullman, 1993; Barker et al.,

1997;Barker et al., 1998) and other ligands (such as siderophores) (Welch et al., 2002;Maurice et al., 2009), which influence saturation, oxidation and reduction of minerals (Newman and Banfield, 2002;Dong, 2010) (summarized in Figure 1.4). In addition, these interactions can also lead to the biomineralization and formation of different minerals (Konhauser et al., 2008).

This direct acceleration of element release influences the saturation and oxidation states of several minerals. Microbial **oxidation or reduction of minerals** occurs in a number of environments at both neutral and acidic pH (Dong, 2010). Subsurface chemolithoautotrophs depend on H_2 , H_2S , S , CH_4 , CO , and Fe^{2+} or Mn^{2+} in rocks as potential electron donors, while CO_2 , Fe^{3+} and Mn^{4+} in rocks or SO_4^{2-} and O_2 in circulating fluids serve as electron acceptors (McLoughlin et al., 2007). **Microbial reduction of minerals** in anaerobic environments is done by microorganisms using metals, such as Fe^{3+} and Mn^{4+} as electron acceptors which are called dissimilatory metal-reducing prokaryotes. The mineral reduction leads to changes in the valence state of these elements which provides energy for the cells. Energy released through these redox reactions depends on the nature of the metal, products and the metabolic pathway employed (Chapelle, 2001).The most studied in the context of microbial reduction include iron and manganese oxides (Kappler and Straub, 2005) as well as within phyllosilicates (Dong, 2010). The rate and extent of microbial reduction of Fe(III) and Mn(IV) in oxides and phyllosilicates are generally correlated with mineral composition, the extent of bioreduction, solution chemistry, the nature of microorganisms involved, crystallinity, surface area, and redox potential where the most crystalline minerals are the least reducible (Croal et al., 2004;Benzerara et al., 2005;Hastie et al., 2007). Microbial reduction of Fe(III) and Mn(IV) oxides often results in dissolution of the minerals and release of other adsorbed or structurally incorporated elements. In addition, also bioreduction of Cr(VI) to Cr(III), U(VI) to U(IV), and Tc(VII) to Tc(IV) , As(V) to As(III), and V(V) to V(IV) has been described before, which typically results in precipitation of these metals (Lovley, 2000). Isolates, capable of reducing minerals, belong to the *Bacteria* or *Archaea* and possess diverse physiological characteristics ranging from psychrophilic to thermophilic as well as facultative to obligate anaerobic metabolisms. Two mostly intensively studied genera of bacteria are *Geobacter* and *Shewanella* (Pennisi, 2002). The current understanding of this interaction, is that microbial reduction of metal elements in minerals can be

accomplished via six possible mechanisms: (1) direct contact (Vaughan and Lloyd, 2011), (2) the presence of natural humic substances and related compounds which serve as electron-shuttling compounds (Nevin and Lovley, 2002), (3) soluble shuttles produced or present in the bacteria themselves (redox active organic compounds which contain quinone moieties, cytochromes, dehydrogenases and secretory proteins mostly present in the outer membranes of the bacteria) which may contact the mineral surface directly (Marsili et al., 2008), (4) nanowires (Reguera et al., 2006), (5) EPS, such as polysaccharides (Marshall et al., 2006), and (6) chemotaxis (Childers et al., 2002). Another mechanism is **microbial mineral oxidation** in which metal sulfides are oxidized. This can have an important economic application i.e. recovery of Au and other trace metals from sulfides and mine spoils (Hao et al., 2010). *Acidithiobacillus ferrooxidans* is widely studied and is one of the few microorganisms known to gain energy by the oxidation of ferrous iron as well as reduced sulfur compounds and these processes are applied within biomining operation (see section biomining). This organism thrives within acidic environments, using the low pH of its natural environment to generate reverse electron flow from Fe(II) to NADH (Valdés et al., 2008).

For indirect bioweathering and release of elements, fungal and cyanobacterial endoliths in the upper rock phases release **acids to dissolve rock** and deploy calcium pumps to sequester calcium ions, which impacts the dissolution of rock carbonates (Garcia-Pichel, 2006). Leaching of calcium and magnesium was enhanced by the cyanobacterium *Nostoc punctiforme* and ascomycete *Knufia petricola* from carbonate and silicate minerals (Seiffert et al., 2014; Ng et al., 2016). Minerals are known to be susceptible to various biological by-products of bacterial metabolism, including protons, organic acids and more complex molecules. A significant body of work has focused on mineral dissolution in the presence of **organic acids** such as citrate, oxalate and gluconic acid. Concentrations of organic acids in soils are difficult to measure (Banfield et al., 1999), but some reported values for bulk soils include 1.5–3.0 x10⁵ M (Hausrath et al., 2009). Some minerals which have been dissolved in the presence of citrate include feldspar, quartz, augite, muscovite, kaolinite, illite, hornblende, apatite and phosphate rocks. Gluconic acid, which harbours both acidifying and chelating functions, is a metabolite frequently reported for its ability to induce phosphate solubilization (Welch and Ullman, 1993). Mineral dissolution can

also be due to carbonic acid, nitric or nitrous acid produced by respiring and nitrifying bacteria (Barker et al., 1997). The effect of ligands on dissolution may be partly due to the direct effect of the ligands on the mineral surface, whereby they polarize and weaken the bond between the cation and mineral lattice (Stumm et al., 1983). However, indirect mechanisms have also been proposed, whereby the complexation of the ion in solution indirectly affects the dissolution of the mineral surface (Oelkers and Schott, 1998). Organic acids and chelating molecules have a triple action on mineral weathering: (i) they adhere directly to mineral surfaces and extract nutrients from mineral particles by electron transfer; (ii) they break the oxygen links; and (iii) they chelate ions present in solution through their carboxyl and hydroxyl groups, indirectly accelerating the dissolution rate of the mineral by creating an imbalance between cation and anion concentrations in the solution (Welch et al., 2002). Another major mechanism involved in mineral weathering is acidification. Both acidification and complexation can be used simultaneously by bacteria to impact mineral stability. *Agrobacterium* and *Bacillus* strains were already described for their ability to weather phlogopite via aluminum chelation and acidic dissolution of the crystal network (Leyval and Berthelin, 1989). Production of gluconic acid has been reported for a strain of *Burkholderia* (Kim et al., 2005). There is a heterogeneity among bacteria which mechanisms or combination of mechanisms are used, i.e. certain isolates are more active in mineral solubilization when xylose or glucose are available, whereas the weathering activity of other bacteria is increased in the presence of lactose or mannitol (Uroz et al., 2009).

Next, **siderophore** production can impact mineral dissolution (Newman and Banfield, 2002). Siderophores are low-molecular-weight organic ligands that form strong complexes with metals and radionuclides, mostly in aerobic environments (Maurice et al., 2009). Many aerobic microbes can produce siderophores as a means to extract essential nutrients from minerals. *Pseudomonas mendocina* can produce siderophores to extract iron from goethite, hematite, ferrihydrite, and kaolinite (Maurice et al., 2001). The stability of siderophore-Fe(III) complexes is extremely high (Kraemer et al., 2005). However, siderophores and other ligands have been shown to form not only stable complexes with iron but also with other metals including Mo, Cu, Co, Mn and Al (Gadd, 2010).

In addition to microbial dissolution of minerals (Dong et al., 2003), microbial activity also contributes to the formation of different minerals, in a process termed **biomineralization** (Konhauser et al., 2008). Oxides, sulfides, and carbonates are typical examples of biogenic minerals (Lian et al., 2006). Within biomineralisation two different processes are separated from one another. One is that of **bacterial controlled mineralization (BCM)** where certain microorganisms exert a genetic and biochemical control over the nucleation of these minerals (Dong, 2010). These biogenic minerals may exhibit a typical morphology, structure, and chemical and isotopic composition that may be used as biosignatures (Benzerara et al., 2005). The minerals produced by bacteria through BCM are usually well-ordered crystals with narrow size distributions, specific particle morphologies, and chemical purity (Dove, 2010). Previous studies have focused on intracellular magnetite formation by magnetotactic bacteria, called magnetosomes, as typical example of biologically controlled mineralization (Frankel and Bazylinski, 2003). These bacteria can also form other intracellular precipitates, i.e., Fe-, As-, Mn-, Au-, Se-, and Cd-precipitates, but the biological and environmental function of these magnetosomes remains unclear (Edwards and Bazylinski, 2008). Other studies also focus on the intracellular formation of iron and manganese granules by *Shewanella putrefaciens* strain CN32 as this bacterium can reduce Fe(III) into ferrihydrite and Mn(IV) into birnessite or pyrolusite (Sand et al., 2001). The other is that of **biologically induced mineralization (BIM)**, where microbes precipitates minerals but these processes are not as controlled as in BCM. In BIM microbes form minerals through alteration of local pH and removal or complexation of certain minerals on their cell surface, while these living cells themselves can provide nucleation sites for mineral precipitation (Konhauser et al., 2008; Dove, 2010). The cell surface has certain functional groups, mainly carboxyl, hydroxyl, amine, and phosphate groups which are negatively charged and thus attract positively charged metal cations from the natural environments (Beveridge and Murray, 1980). These precipitates are usually amorphous, but with time, they undergo transformation to more crystalline minerals. Many types of minerals have been observed on cell surfaces, including (hydr)oxides, silicates, amorphous silica, carbonates, phosphates, sulfates, and sulfides (Konhauser et al., 2008). Studies have also investigated biogenic precipitation of CaCO_3 and the microbial formation of carbonates (Ehrlich and Newman, 2009). These early studies demonstrated that CaCO_3 precipitation was not specific to any group of bacteria and depended on

environmental conditions. The precipitated carbonates can be formed via diverse mechanisms; aerobic or anaerobic oxidation of carbon and nitrogen compounds, reduction of sulfate to sulfide, hydrolysis, and removal of CO₂ from bicarbonate-containing solution (Ehrlich and Newman, 2009). More recent studies, have shown that carbonate precipitation also occurs in marine environments (the Bahamas and the Gulf of Mexico), caves and saline lakes/lagoons (Dong, 2010; Miot et al., 2014).

1.2.1.2 Biofilm formation

The major part (99.9%) of the microbial biomass present in the subsurface has been estimated to be part of the biofilm attached to surfaces (Madigan et al., 2008). The definition of the term “biofilm”, given by Characklis and Marshall (1990) is the following: “immobilized cells attached to solids (such as minerals), growing, reproducing and producing extracellular polymers which frequently extend from the cell forming a tangled matrix of fibers which provide structure to the assemblage termed a biofilm”.

Colonization and microbial attachment of microbes on mineral surfaces, due to chemotactic processes (Gorbushina and Broughton, 2009), leads to the production of extracellular polymeric substances (EPS). Surface appendages may play an important role within attachment, as they serve as anchors to solid surfaces and can induce subsequent mineral dissolution (Welch et al., 1999). Some surface appendages can also conduct electricity and may represent a common bacterial strategy for efficient electron transfer under anaerobic conditions (Ntarlagiannis et al., 2007). The attached cells tend to produce signal molecules to attract other organisms to gradually build the biofilm community (Harrison et al., 2005b). It has also been shown that biofilm bacteria form a micro-environment on the mineral, as for example on pyroxenes, by inducing an amorphous Al-rich layer beneath the microorganism, as well as calcium carbonates precipitations which associate with the EPS adjacent to the microorganism (Benzerara et al., 2005). EPS increases mineral weathering through chelating metals as well as protonation on the mineral surface. It was demonstrated that once a EPS matrix forms, it also absorbs water and prevents dessication due to its hydrophilic properties (Papida et al., 2000). Fluidic conditions maintained within the biofilm are also very different to those in the surrounding environment outside the biofilm (Vaughan and Lloyd, 2011).

The biofilm manner of microbial life has a major impact on cell physiology and growth (Stoodley et al., 2002). Biofilm dwelling has several advantages for the microbial cells as mineral and rock surfaces can provide sufficient supplies of nutrients and allow

protection from lethal environmental stress and predation. However this environment can be heterogeneous with microscale variations in surface chemistries that can impact community composition: i.e. bacterial biofilm communities present on similar surface types (carbonates, silicates and aluminosilicates) were shown to be more taxonomically similar (Jones and Bennett, 2014;2017). In addition, the metabolic potential of mineral-associated microorganisms in biofilms differs from those in the surrounding soil, which are not associated with the biofilm, and a higher metabolic potential was observed in organisms associated with the smaller rather than the larger mineral fractions present (Certini et al., 2004). Research into the genetic and proteomic changes within bacteria dwelling in biofilms on rocks has shown that cells within the biofilm showed differential protein expression profiles when forming biofilms on minerals (Vera et al., 2013).

The biofilm manner of microbial life has also a major impact on the environment associated with the biofilm (Stoodley et al., 2002). Once a biofilm community is established, minerals and rocks undergo weathering, i.e., some minerals dissolve, some precipitate, and some undergo transformation (Dong, 2010). The development of biofilms can also have a significant impact on the flow of fluid through fractures in rocks or through porous media, causing so-called bio-clogging (Brydie et al., 2009). Different variables such as grain size, oxygen availability, organic carbon availability impact bio-clogging and the mobility and precipitation of minerals and metals such as copper or iron hydroxides (Boult et al., 2006).

1.2.1.3 Basalt-microbe interactions

The prominent role of microorganisms in the weathering of basalt and glass has been demonstrated in both laboratory culture study (Staudigel et al., 1995; Thorseth et al., 1995a; Staudigel et al., 1998; Bach and Edwards, 2003; Wu et al., 2007) and in situ incubation experiments (Bach and Edwards, 2003). Microbes can influence the dissolution of olivine, pyroxene (Welch and Banfield, 2002; Benzerara et al., 2004; Josef et al., 2007; Wu et al., 2007; Santelli et al., 2009).

Microorganisms at and beneath the **ocean** floor play an important role in rock/glass alteration (Fisk et al., 2006; Furnes et al., 2007; Staudigel et al., 2008) and chemical and isotopic exchange between the oceanic crust and the sea water (Fisk et al., 2006), impact biogeochemical cycles of C, Fe, S, and other elements (Edwards et al., 2005). Ocean floor basalt contains sufficient quantities of reduced Fe and S to provide

chemical energy that can support biomass formation. Fe(II) oxidation is an important process in oceanic basalt alteration (Henri et al., 2015). In addition, the presence of Mn(II)-oxidizing bacteria can also indicate that Mn(II) oxidation is an alternative route of energy generation (Templeton et al., 2005; Staudigel et al., 2008; Smith et al., 2011). Fe- and Mn-oxidizing lithoautotrophs isolated from deep sea environments have been demonstrated to be capable of growth on basalt glass as the sole source of energy and they are found to enhance the rate of basalt dissolution by up to an order of magnitude (Templeton et al., 2005; Edwards and Bazylinski, 2008). In these processes, they predominantly derive energy from the oxidation of H_2 , ferrous iron, manganese, sulfides and methane as well as the fermentation of organic matter. It has been shown that the bacterial activity has a specific impact on basalt alteration, due to changes in pH which result in localized dissolution of the glass. These pH differences may vary, depending on the type of bacteria or consortium as well as the presence of rock-structuring elements such as Si and Al (Thorseth et al., 1992). These specific basalt 'bio-alteration' leaves distinct textural and geochemical features which lead to deformation of the basaltic rocks. This bioalteration has been shown in pillow lavas both on continental as well as deep ocean deposits (Staudigel et al., 2008; McLoughlin et al., 2009). The ocean drilling program showed that for the seafloor basalt, biological alternations in this environment dominates over the abiotic weathering of the glass (Furnes et al., 2007).

In **terrestrial basalt** environments, bioweathering by phototrophic organisms, mostly cyanobacteria, is of particular importance because they initially colonize newly exposed rock surfaces (Büdel, 1999; Crispim and Gaylarde, 2005) and a link between photosynthetic micro-organisms, increase in pH and bioweathering, has been suggested (Büdel, 1999). This increase in the micro-environmental pH is directly caused by photosynthetic metabolism (Thompson and Ferris, 1990). Experimental work with *Synechococcus* showed that an increase in pH is due to the fact that cyanobacteria use HCO_3^- as primary carbon source and exchanging this for OH^- (Miller and Colman, 1980). This will increase the dissolution of some of the key rock-forming silicate components, such as feldspars, biotite, as well as mafic and felsic interstitial glass (Chou and Wollast, 1984; Malmström and Banwart, 1997; Gislason and Oelkers, 2003; Wolff-Boenisch et al., 2004). Abiotic weathering studies with crystalline basalt have shown that minimum elemental release rates occur at neutral pH (between pH 6

and 7 depending on temperature), but that both basic and acidic conditions accelerate elemental release (Gudbrandsson et al., 2008). The released elements are utilized by the cyanobacteria, causing an increase in specific growth rate and photosynthesis activity, which accelerates rock dissolution. However, not all minerals will respond to these pH changes in the same way. In contrast to interstitial glass, the dissolution rates of other, principally basaltic constituent minerals, such as olivines and pyroxenes, do not increase at high pH (Oelkers and Schott, 2001). In these cases, increased elemental release rates from the basaltic medium may be accounted for through improved access to these key minerals by the overall pH-enhanced dissolution of plagioclases and, if present, interstitial glass (Olsson-Francis et al., 2012). Wu et al. (2007) showed that *Burkholderia fungorum* activity substantially elevated the release of Ca, Mg, Si, and S compared to abiotic conditions which impacted and accelerated the release of apatite bound P. Conversely other work has shown that microbial activities can inhibit elemental release by facilitating development of an amorphous leached layer, promoting adsorption of polysaccharides onto mineral surfaces, preventing formation of etch-pits (Welch et al., 1999; Lüttge and Conrad, 2004; Benzerara et al., 2005), and releasing ferric iron that interacts with available surface sites (Welch and Banfield, 2002; Santelli et al., 2009).

1.2.1.4 Biomining

The use of micro-organisms to facilitate the extraction and recovery of precious and base metals from primary ores and concentrates, generically referred to as 'biomining', has developed into an effective and expanding area of biotechnology. Microbial oxidation and leaching is commonly used for the recovery of metals, such as Au and Cu, in sulphide minerals. These metals can be released from refractory minerals by oxidation of the covalent metal-sulfide bond (Uboldini et al., 2000b). After bio-oxidation, the initial insoluble metal sulfide is then totally or partially dissolved, and most of precious metals within sulfides can be extracted in the subsequent extraction processes. In these bio-oxidation processes, the microorganisms are added to the leaching solution (Dong, 2010). This is in contrast to acid rock drainage where bacteria can be used to counteract the formation of acidic or alkaline effluents formed during abiotic mining due to exposure of the mining effluent to oxygen and water. Here, sulfate reducing bacteria can be used to remove metals and sulfate (Panda et al., 2016).

The most common microorganisms involved in the bio-oxidation mining processes are bacterial species of the genera *Acidithiobacillus*, *Leptospirillum* and *Sulfolobales* species as well as representatives of the Archaea genus *Ferroplasma* (Sand et al., 2001). Microorganisms, isolated from different environments, which have a good potential for bio-oxidation mining include: *Acidiphilium cryptum*, *Metallosphaera sedula*, *Acidimicrobium ferrooxidans*, *Leptospirillum ferrooxidans*, *Ferroplasma*, *Sulfolobus metallicus* and *Acidianus brierleyi* (Holmes et al., 2009). Molecular studies described the mechanisms behind these microbe-mineral interactions (Valenzuela et al., 2006; Jerez, 2008; Holmes et al., 2009) and demonstrated the importance of lateral gene transfer in increasing genetic and metabolic potential of these biomining organisms to survive and act in such environments (Brierley and Brierley, 2001; Holmes et al., 2009). Combining culture dependent and independent analyses, Okibe and Johnson (2004) identified the most efficient microbial consortium for pyrite oxidation: *Acidithiobacillus ferrooxidans*, *Acidithiobacillus caldus* and *Leptospirillum ferriphilum*. These communities enhanced pyrite dissolution compared to pure bacterial cultures (Mapelli et al., 2012). Several species of fungi can also be used for biomining; two fungal strains *Aspergillus niger* and *Penicillium simplicissimum* were able to mobilize Cu and Sn with a 65% yield as well as Al, Ni, Pb and Zn with a yield of more than 95% (Brauer, 1990).

Biomining is responsible for approximately twenty percent of global copper and five percent of global gold production as well as smaller amounts of iron, nickel, chromium, cobalt and manganese. It has evolved from irrigated and aerated heaps to temperature-, aeration- and pH-controlled stirred tanks (Johnson et al., 2013; Klas et al., 2015). Dump and heap reactors are still typically used for leaching low-grade, run-of-mine rock that would otherwise be discarded (used i.e. for copper ores) (Tuffin et al., 2006; Rawlings and Johnson, 2007). Biomining has distinctive advantages over the traditional mining procedures. It does not require the high amounts of energy used during roasting and smelting and does not generate harmful gaseous emissions such as sulfur dioxide. Nevertheless, toxic metals such as lead and arsenic can be generated, which if not properly controlled, pollutes the environment. Biomining is also of great advantage since discarded low-grade ores from standard mining procedures can be leached in an economically feasible way (Valenzuela et al., 2006; Rawlings and Johnson, 2007).

1.2.2 The space environment and application of microbe-mineral interactions

1.2.2.1 Exploration and biological ISRU

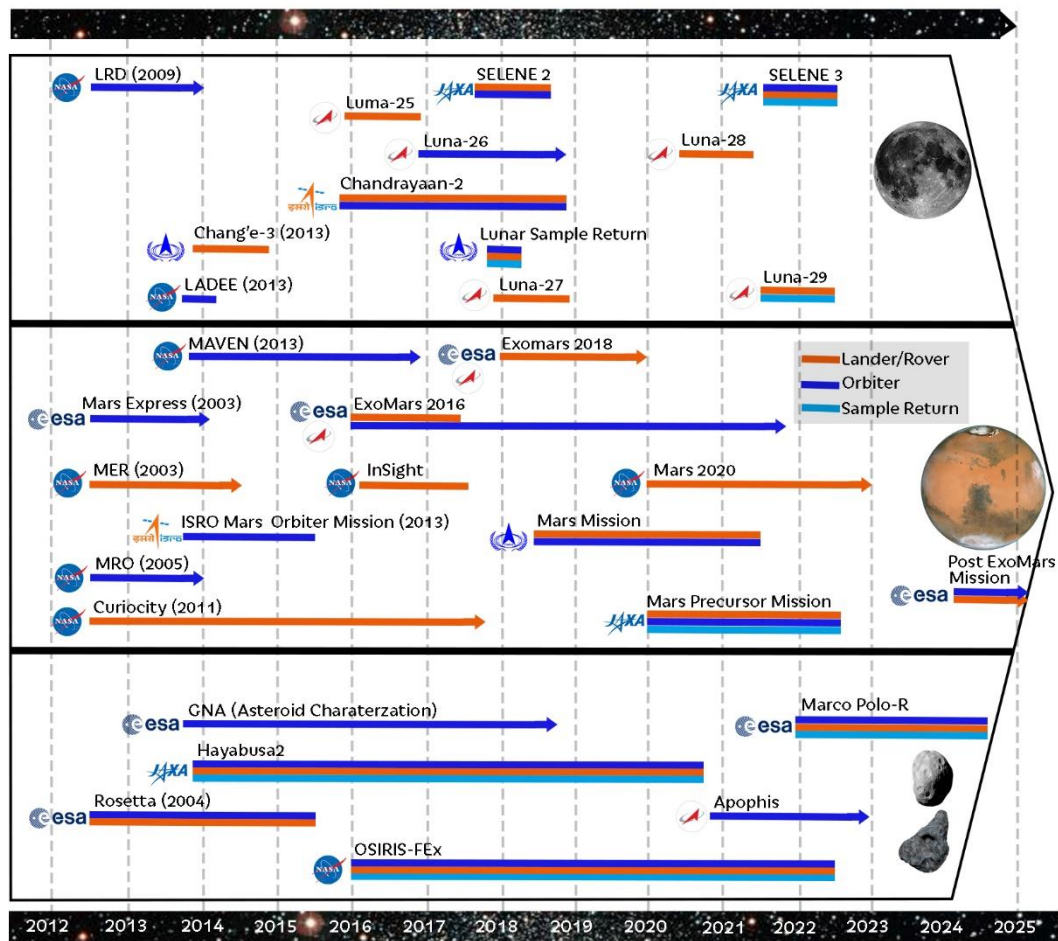


Figure 1.5: Past and future planned space missions, based on Astronoo (2014)

The National Astronautics and Space Administration (NASA) aims at having technologies ready at the end of the 2030's in order to send and support a human presence on the surface of Mars. Although still little is known about their plans, its deep space crew capsule successfully made its first in-space test on December 5, 2014 (Figure 1.5). Last year Elon Musk, CEO of Space Exploration Technologies Corporation (SpaceX), announced that he targets a manned Mars mission in the next 50 years. Further development is necessary on reducing costs as now, varying on different mission scenarios, launch costs would be in the order of \$300 000 per kilo for Mars (Massa et al., 2007). In terms of amounts of dietary supplies, similar to what is available on ISS, about 1.8 kg/day per crewmember is needed which would results in 29 tons of food, which could supply a 6 member crew for one year. However, the amount of supplies necessary for a healthy diet would even be higher (Allen et al.,

2003;Verseux et al., 2016a). In terms of oxygen demand, 1 kg of O₂ per day needs to be supplied (Allen et al., 2003).

New bioregenerative life support systems are or have been development to convert solid, liquid and gaseous wastes produced by the human crew into nutritional biomass, oxygen and potable water both in space and within lunar and martian outposts (Gitelson, 1992;Nelson et al., 2010). As such the crew would become more independent of Earth supplies and more selfsustainable. The European Micro Ecological Life Support System (MELiSSA) is a model system that is being developed by the European Space Agency (ESA) as an advanced life support system, based on different microbial species and higher plants. The system consists of a number of bioreactors and a higher plant chamber, which break down the organic waste from the crew and non-edible parts of the plants. Each of the recycling step has been inspired by the natural organic waste reconversion cycle taking place in a natural lake ecosystem (Hendrickx and Mergeay, 2007). Similar programs exist all over the world, such as the Controlled Ecological Life Support System (CELSS) program and the closed biosphere BIOS-facilities (Bios 1, 2 and 3) in the US, and the Closed Ecology Experiment Facility in Japan. Multiple growth tests have been done with plants in the growth facilities on the space stations (Mir and ISS) (Lehto et al., 2007). These experiments with higher plants showed that plants are capable of long-duration normal growth, full development and reproduction without deviations under real space flight environment (Sychev et al., 2008). Thus in principle, biologically, it could be possible to cultivate food in space. However, while space travel technologies have made great steps forward in the last half century, current life support technologies are not sufficiently developed for sustaining Mars manned exploratory missions (Horneck et al. 2006).

Today, two of the most important constraints on the successful establishment of stations on other planetary bodies are still the complexity and cost of transporting consumables out of Earth's gravity and across interplanetary space. The use of local resources to sustain a human presence could therefore be both logistically and economically desirable. In Situ Resource Utilisation (ISRU) may drastically reduce mission payload, cost, and risk for exploration and prolonge the maximal duration of future missions. Physicochemical based ISRU can be an important part of future life support but so is biobased ISRU. Some products such as high-protein food can

currently not be produced or recycled without biological processing (Drysdale et al., 2003; Montague et al., 2012). A lot of our daily products used today are produced by organisms: foods such as yoghurt, drugs, various chemicals, biomaterials, biofuels and mined metals (Verseux et al., 2016b). Microorganisms can not only be used to extract and biomine useful elements on Earth but could also be used to extract useful elements from extraterrestrial materials for industrial processes or as nutrients in life support systems. In addition, microorganisms could be used to create soil from lunar and Martian rocks.

Present geomicrobiology research on Earth is focused on the use of microorganisms to leach or mine metals of industrial interest (e.g., copper, nickel and gold) from rocks, and their use on Mars to mine basalt and potential ores has been suggested (Cockell, 2010). Extracted elements could be used within an outpost for many chemical and manufacturing processes such as carbon dioxide cracking, electroplating, production of alloys and manufacturing of solar cells (Cockell, 2011). Microorganisms could be used for improving building materials such as bioplastics and concrete-like materials (McKay and Marinova, 2001; De Muynck et al., 2010; Hempel et al., 2011; Verseux et al., 2016a; Verseux et al., 2016b). Bacterial produced three-dimensional printed PHB blocks could also be used for habitat and furniture construction (Menezes et al., 2015a). In addition, bacteria could also be used to minimize dust, which is a severe hazard as the Moon or Mars regolith is subject to long-term pulverization through impact events, resulting in the release fine dust and small fragments (Kahre et al., 2006). DeJong et al. (2006) showed how carbonate producing bacteria can be used to solidify ground by binding mineral grains in carbonates by using i.e. *Bacillus* sp. A second approach to binding surface material is to bind regolith minerals together using the 'matting' habit of microorganisms (Liu et al., 2008). This approach uses the tendency of some microorganisms to grow across the surface of substrates and bind grains within the polysaccharide exuded by these organisms. This could also be used within enclosed spaces as a filter, removing dust particles entering the habitat (Cockell, 2011). Research has also been shown that bacteria could be used within microbial fuel cells (MFCs) which could generate electricity and process waste sludge and waste water (Jiang et al., 2009). Martian basalt could be mixed with wastewater or solid waste and thus chemically be broken down to release nutrients for the biota. It could be used as a source of oxidized iron for the iron-reducer *Geobacter* spp. that would be used to

achieve the degradation of organics in habitat wastewater and solid waste (Cockell, 2010). Cyanobacteria could also be used to directly convert solar energy into biofuels: they can produce various energetic compounds such as alkanes and lipids as well as dihydrogen which could be used to reduce locally available CO₂ to hydrocarbons and produce fuel (Hepp et al., 1993; Quintana et al., 2011; Raksajit et al., 2012). Yeasts are efficient ethanol producers but will rely on cyanobacterial biomass or sugars secreted by cyanobacteria as carbon sources (Möllers et al., 2014). For a mission to Mars, the initial mass launched from low Earth orbit may be reduced by 20–45% if hydrocarbon propellants are used. In addition, antibiotics, therapeutic peptides, antioxidants and other nutraceutical compounds can be produced with the help of microorganisms or microbes could be engineered to contain new metabolic pathways leading to the production of various drugs. The most famous example is the production of a direct precursor of artemisinin, an antimalarial drug in yeast and bacteria (Ro et al., 2006). In a final step, organisms could also be used to perform terraforming. The Biosphere 2 project attempted to create a materially self-enclosed system, capable of supporting eight humans for two years. The project discovered that its fertile carbon-rich soils together with the presence of microorganisms were essential for plant photosynthesis, while the initial biodiversity was lost over time if not actively controlled. Furthermore, due to failure of the system, the biosphere 2 crew showed impaired caloric and nutrient intake (Walford et al., 2002).

Due to the harsh conditions present on all space bodies, microorganisms should possess sufficient robustness to be transported in an inactive state to these locations, and to be able to tolerate equipment failure and exposure to unshielded environmental extremes (Holladay et al., 2007). Synthetic biology could be complementary to the applications mentioned above and used to increase the resistance of microorganisms to Martian conditions, to reduce both hardware needs and risks of culture loss. Of particular interest would be to increase resistance to long-term dehydration, to radiation, to a wide range of temperatures, to low pressures and to large shifts in these parameters as well as resistance to multiple of these parameters (Verseux et al., 2016b). In addition, research is focussed on ways of engineering microorganisms to increase their abilities to extract (and possibly sort) elements of interest. Cyanobacteria have been extensively studied for their use within ISRU. This has been excellently reviewed by Verseux et al. (2016a). It was shown that cyanobacteria, isolated from

rock- dwelling community, could survive in Mars simulated conditions. Some of the tested species survived 28 days in desiccated state exposed to Mars stimulation conditions. Some were even able to grown when incubated with Martian and lunar analog basalt, rhyolite and andorthosite. The growth rate and rock dissolution were significantly lower with rhyolite demonstrating the importance of silica content for ISRU. Biological weathering resulted in the release of bio-essential elements from the rock matrix (Olsson-Francis and Cockell, 2010b).

1.2.2.2 Bacterial behaviour in space

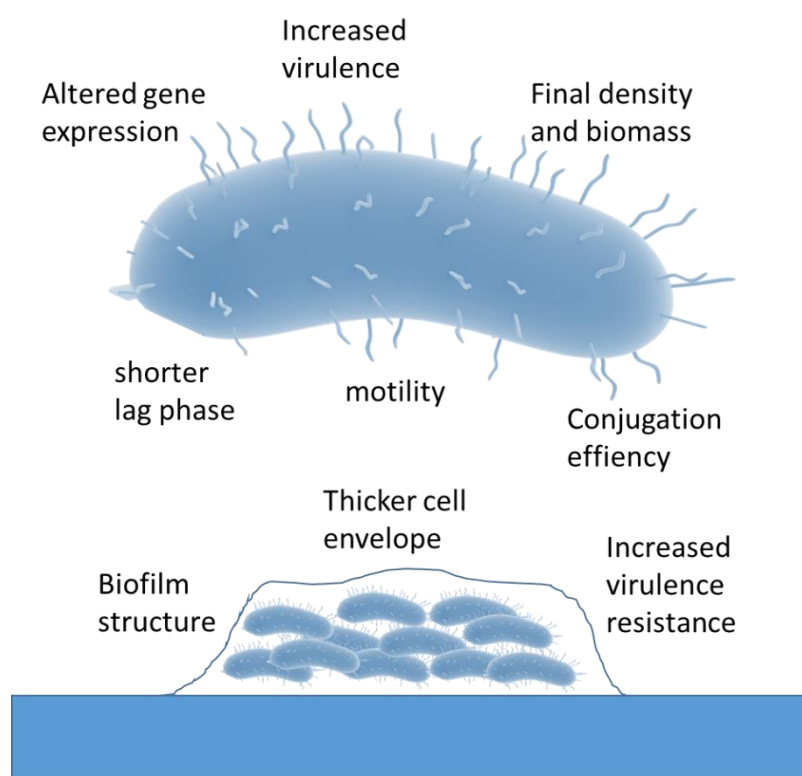


Figure 1.6: Space impact on different parameters of bacterial life and bacterial biofilm formation.

Although microbe mineral interactions could have several application in space, the impact of space on microbe-mineral interactions has not been investigated before (Figure 1.6).

Although many species of prokaryotes have been studied in ground simulation facilities (which have been summarized by Olsson-Francis and Cockell, 2010) or have been flown in space flights, the large variation with regard to culture conditions, individual response of bacterial species, procedures and the use of equipment, have made it difficult to draw general conclusion on the impact of space conditions on microbes (Leys et al., 2004; Wilson et al., 2008; Olsson-Francis and Cockell, 2010a; Kim et al.,

2013a). Nevertheless, in general, simulated microgravity in ground-based experiments, as well as flight experiments, induced several effects on active bacterial cultures which can be summarized as following: 1) altered production of secondary metabolites; 2) global alteration of gene expression induced by adaptation to simulated microgravity, possibly leading to difference in behavior in space flight; 3) increased virulence; 4) increased final cell density; 5) antibiotic resistance; 6) shorter lag phase; 7) increased biomass; 8) a thicker cell envelope and 9) enhanced conjugation efficiency (Leys et al., 2004; Kim et al., 2013b; Taylor, 2015; Zea et al., 2016). The results of previous microgravity experiments on ISS has been summarized in the review of Horneck et al. (2010). However, long term observations of more than 6 months are still rare. It is still to be elucidated how weightlessness causes these effects and how both molecular as well as physiological changes will be impacted during long term missions. The major factor leading to these differences is due to the microgravity, but bacteria are also exposed to vibration, and acceleration during spaceflight. Radiation in addition, may also increase bacterial mutation rates i.e. when mutation rates were compared within one cloned bacterial gene within *Saccharomyces cerevisiae*, it was shown that it contained two to three times more mutational differences after 40 days of space flight compared to ground (Fukuda et al., 2000). Microgravity leads to diffusion becoming the predominant way for transporting nutrients to and waste away from the cells, in comparison to convection, and inhibits settling of cells, buoyancy, and sedimentation. As similar growth kinetics were observed of bacteria on solid media compared to ground, it indicates that these effects are solely due to diffusion. Molecular studies and the effect of motility on growth of bacteria have confirmed these findings as motile cells can move towards more preferential microenvironments supporting survival and growth as well as mix the cellular layer present around the cell counteracting the inhibition of convection (Taylor, 2015; Zea et al., 2016).

The effect of spaceflight on microbial communities and within **biofilm** has been much less investigated. As most studies consist of short span studies in suspension cultures, this does not represent the biofilm dwelling way of bacteria. Nevertheless, the formation of biofilms in space is a relevant research topic. It is known, from Earth applications, that biofilms exhibit higher resistance against stressors such as antibiotics (Stewart and Costerton, 2001). Controlling of biofilm formation in space

aircraft using conventional antimicrobial techniques is thus a problem. For example, large biofilms were detected on the Russian Mir station causing blocking of the drinking water purification system (Gu et al., 1998). Some biofilm studies in space have shown that *E. coli*, *Salmonella* and *Pseudomonas* can form more biofilm during space flight compared to ground controls leading to increased antimicrobial resistance, increased cellular clumping and aggregation. *Pseudomonas* was shown to form a biofilm with a column-and-canopy-shaped architecture which was influenced by flagella-driven motility (Kim et al., 2013b). Both nutritional composition as well as phosphate and oxygen levels impact biofilm growth, virulence and final biomass during spaceflight (Rosenzweig et al., 2014).

Today, little to nothing is known on the impact of space conditions on biofilms on minerals and microbial rock weathering. Expose facilities on the ISS did allow studying survival of organisms, organics and other astrobiology relevant molecules, in contact with rocks and minerals. Therefore, the Expose-E facility was installed outside the Columbus facility on ISS which provided accommodation for a variety of astrobiological test samples that were exposed to selected space conditions during 1.5 years: space vacuum, solar radiation and cosmic radiation and simulated Martian surface conditions. All biological samples within the Lichens and Fungi Experiment (LIFE) were rock-dwelling organisms isolated from hostile regions: Antarctic cryptoendolithic communities present in sandstone, microcolonial black cryptoendolithic fungi (*Cryomyces antarcticus* and *Cryomyces minteri*) also isolated from Antarctic sandstone, and high mountain epilithic lichens (*R. geographicum* and *X. elegans*) (Sancho et al., 2007; de La Torre et al., 2010). The cryptoendolithic communities and black fungi from the Antarctic desert survived during the duration of the EXPOSE experiment and thus exposure to high vacuum, temperature fluctuation, the full spectrum of extraterrestrial solar electromagnetic radiation, and cosmic ionizing radiation. These communities could in addition also survive under simulated space and Martian conditions in ground-based Experiment Verification Tests (Onofri et al., 2012; Rabbow et al., 2012). But these tests, were mainly focussed on survival of the organism in the extreme outer space conditions with the help of the mineral, not the active bio-weathering process of the minerals which could (and likely should) be performed in the shelter of a bioreactor or habitat structure.

1.2.2.3 Societal implications of the application of ISRU

In addition to the obvious technical challenges, the development of lunar (and other extraterrestrial) resources will require the establishment of an international legal regime. Such regime should encourage large-scale investment in prospecting and extraction activities, while at the same time ensure that space does not become a possible flashpoint for international conflict and actors keep to planetary protections regulations. Currently space activities are governed by a small set of international treaties negotiated by the United Nations (UN, 1984). The most important of these, and the foundation on which the international legal regime for outer space is built, is the 1967 *Treaty on Principles Governing the Activities of States in the Exploration and Use of Outer Space, including the Moon and Other Celestial Bodies*, more commonly known as the Outer Space Treaty (OST). The OST was intended to establish a number of important ‘principles’ for the activities of nation-states in space. These include the concept that space should be considered “the province of all mankind” (Article I), that outer space is free for the ‘exploration and use’ by all states (Article I), that the Moon (and other celestial bodies) cannot be appropriated (by claim of sovereignty or otherwise) by nation-states (Article II), and that international law, including the UN Charter, applies to outer space (Article III). Thus, the Moon, Mars and other celestial bodies, cannot be used for the economic exploitation of resources (Klas et al., 2015) as it prohibits governments from extending sovereignty or claiming resources. For the past 50 years, however, most of our space activities have been focused on Earth orbit. Only recently there has become support about guidelines and regulations necessary to make exploration possible as well assess the impact of commercial and private activities on the exploration of Lunar, Martian, or other celestial bodies. Therefore COSPAR has updated their Planetary Protection Policy in August 2015 (Kminek and Rummel, 2015). Planetary protection can be divided to two different areas, the first is that of protecting extraterrestrial environments from terrestrial contamination (forward contamination) while the second deals with protecting the Earth from contamination of samples or hardware returning to Earth (back contamination) (Arnould and Debus, 2008). This forward contamination is an important concern for missions to Mars as it will influence future life detection missions and analysis, as well the impacts of finding ‘Martian life’. Depending on mission design and plan, these recommendations thus need to be adjusted (Arnould and Debus, 2008; Olsson-Francis and Cockell, 2010a).

It is thus clear that further steps need to be taken, both in terms of legal context, political issues as well as planetary protection if outposts or ISRU activities would be used to establish a persistent human presence (Crawford, 2015; Carpenter et al., 2016; Rettberg et al., 2016).

1.3 Choice of model organism and rock

As mentioned above, microbe-mineral interactions could also have their use in space exploration as resupply from Earth becomes less evident for more distant future space missions. The European Space Agency (ESA) has established the Topical Team “Geobiology in Space Exploration” to investigate the impact of space conditions on microbe-mineral interactions and to develop potential biobased ISRU applications (Cousins and Cockell, 2016). In this framework, the ESA BioRock experiment, which is currently in preparation, will investigate the growth of bacterial biofilms and element leaching on a lunar rock analogue (basalt) under near weightlessness and Martian gravity (0.38g) within the ISS. For BioRock, three model organisms were selected *Sphingomonas desiccabilis*, *Bacillus subtilis* and *Cupriavidus metallidurans* (Loudon et al., 2017). The latter is the focal point of SCK•CEN’s participation and this PhD.

Cupriavidus metallidurans strains are often isolated from industrial sites linked to mining-, metallurgical-, and chemical industries (Brim et al., 1999; Goris et al., 2001; Diels et al., 2009), and are mostly studied because of their resistance to numerous heavy metals (Goris et al., 2001; Mergeay et al., 2003; Mergeay and Van Houdt, 2015). For *C. metallidurans* type strain CH34, many of these metal resistance determinants are carried by its two megaplasmids pMOL28 and pMOL30 (Janssen et al., 2010; Monsieus et al., 2011) and it has the ability to resist high concentration of transition metals, produce metal-containing minerals, oxidize hydrogen, grow chemolithoautotrophic and degrade a variety of aromatic and organic compounds (Diels et al., 2009; Janssen et al., 2010; Mergeay and Van Houdt, 2014; 2015). The presence of these metal-processing proteins coupled with a broad metabolic diversity makes this organism useful for geomicrobiological and bioremediation studies (Mijnendonckx et al., 2011; Van Houdt et al., 2012; Chalia et al., 2016; Nies, 2016). Their biomineralisation potential has already been shown and used to induce CaCO₃ precipitation for the restoration and preservation of ornamental stones (Daskalakis et al., 2013; Chalia et al., 2016). In addition, CH34 also showed gold accumulation through

Au-regulated gene expression and reductive precipitation of Au^{3+} -complexes. In addition, it also has been shown that CH34 could biomineralize silver and platinum group metals and incorporate them into their periplasm (Diels et al., 1995;Ledrich et al., 2005;Reith et al., 2009;Gauthier et al., 2010). In particular, the interaction of type strain CH34 with basalt showed that stress and starvation responses are triggered in the presence of basalt (Bryce et al., 2016) and that it can sequester iron from basalt to sustain its growth (Olsson-Francis et al., 2010a). Olsson-Francis et al. (2010a) also showed that siderophores were only produced when grown in iron-limited medium and not when basalt was added, showing that *C. metallidurans* CH34 could use the iron directly leached out from basalt. In addition, also its response in space conditions is known and type strain CH34 has been used previously as test organism to investigate its growth behavior in space (Leys et al., 2009) .

Next, closely related species, *Cupriavidus necator* and *Cupriavidus taiwanensis* are model organisms to study poly-3-hydroxybutyrate (PHB) formation and can accumulate PHB up to 90% of their cell dry weight (Chien et al., 2010). Next to polyphosphates (Achbergerová and Nahálka, 2011), these poly-3-hydroxybutyrates (PHB) serve as energy storage molecules. More specifically, PHBs are highly reduced carbon and energy storage compounds and its synthesis is coupled to carbon and nitrogen metabolism. These storage compounds are also involved in cellular energy redox balance as these polymers act as a sink for reducing equivalents (Raberg et al., 2014;López et al., 2015). In simulated microgravity conditions, *Cupriavidus metallidurans* LMG 1195 was shown to yield higher amounts of PHB compared to normal conditions (De Gelder et al., 2009). PHB accumulation can also protect *Ralstonia eutropha* from entering the VBNC state during cold storage (Northup et al., 2011). This VBNC state has been shown to be induced by a variety of stresses such as pH, temperature and has been identified in 85 different species (Ayrapetyan and Oliver, 2016). Bacteria within VBNC, remain viable and active but lose their culturability on media, and start accumulation of these intracellular storage polymers (Pedrós-Alió et al., 1990). In addition, bacteria transitioning into this state become more resistant against different environmental stresses (Bogosian and Bourneuf, 2001;Oliver, 2016). Recovery of these non-culturable bacteria has been difficult to prove, produce and reproduce (Lopez-Amoros et al., 1995;Gasol and Del Giorgio, 2000;Amor et al., 2002a;Falcioni et al., 2008;Hammes et al., 2011;Boi et al., 2015). Some of these

bacteria resuscitate when the inducing stress is removed, while the involvement of other growing bacteria and quorum sensing molecules can be important in reculturability other (Oliver, 2016). The physiological basis for these processes is not yet fully elucidated (Kell et al., 1998; Breeuwer and Abee, 2000b; Epstein, 2013; Oliver, 2016). It was shown that *C. metallidurans* and closely related *Ralstonia pickettii* isolates can survive in mineral water, even in the presence of antimicrobial silver, for almost two years, but also those cells showed a decline in culturability from 10^9 CFU/ml to 10^6 CFU/ml (Mijnendonckx et al., 2013). *Ralstonia solanacearum* also showed this transition into the VBNC state in river water (Álvarez et al., 2008) or when exposed to high concentrations of copper, low temperature or in soil (Grey and Steck, 2001; van Overbeek et al., 2004; Caruso et al., 2005).

Within this study, basalt was used as proxy for lunar or martian basalt. Although major differences exist between the two (mentioned in section 1.1.2.), the composition of terrestrial basalt is closest to that of the lunar mare (Ruzicka et al., 2001). Seven different basalt could be obtained, by several expeditions and collaborations, and were used in this study: four from Icelandic origin (SCK1-4), one from Hawaii (SCK5), one from the Eifel area (Germany) (SCK6) and one from Norway (SCK7) (Appendix 1), which all classify as basalt type according to the TAS diagram.

Scope of the research

Scope of the research

Taking a broad view, the general goal of this work was to generate insight in the interaction between planetary analogue rock and microorganisms. *Cupriavidus metallidurans* CH34 was used as a model organism because of its selection in the ESA BIOROCK experiment, previous research conducted on its presence in the basalt environment and interactions with basalt, its demonstrated ability for biomineralization, detailed knowledge of its genomic organization and metabolism.

Since basalt is a valid proxy for studying lunar or martian basalt, the current knowledge on basalt composition and chemistry, the presence of basalt formations on Mars and the Moon, as well as the microbial life on and its interactions with basalt and its possible applications for future space missions is reviewed in **chapter 1**.

As a first step the influence of different rock compositions on element leaching and bio-weathering processes was determined, as the impact of these processes are still poorly understood. Therefore, different basalts were used throughout this work to determine the effect of basalt composition on bacterial community structure as well as its effect on growth and survival of *C. metallidurans* (**Chapter 2,3,4 and 5**) (Figure 1.7).

As relatively few data is available on the diversity and richness of the communities inhabiting successive and chronological lava flows, and two expedition opportunities arose, we aimed, at first, at identifying the bacterial communities present on a chronosequence of Icelandic basaltic lava deposits (**chapter 2**). The relationship between the bacterial community characteristics and the composition of the lava basalt was examined as this could provide input for studying the role of microorganisms in mediating and facilitating habitability development on f.e. Mars.

Next, we aimed to gain insights into element leaching from (bio)weathering basalts. In a first phase we evaluated the influence of different basalt compositions on element leaching in element-deficient growth medium and subsequent support of *C. metallidurans* CH34 growth (**chapter 3**). In a second phase, we determined the effect of element leaching in non-actively-growing stationary-phase cells via long-term storage experiments with emphasis on energy status (ATP and PHB levels),

culturability and biofilm formation as these strategies could be deployed by bacteria to increase their survival (**chapter 4**).

In **chapter five**, to prepare for a potential application of the ISRU process in space, this long-term storage experiment was also part of the FOTON flight opportunity. Through this opportunity, further research could be conducted to determine the impact of space flight conditions on the long term storage and microbe-mineral interactions of *C. metallidurans* with basalt, as already studied in chapter 4.

To technically support our work, multicolor flow cytometry, which is used throughout the whole work, was validated as a good approach to estimate and assess multiple bacterial physiological features at once (**chapter 6**).

Finally, **chapter seven** summarizes this research and draws final conclusions on the use of bacteria for ISRU, basalt habitability and the effects of different basalt compositions on bacterial physiology and its interactions with basalt.

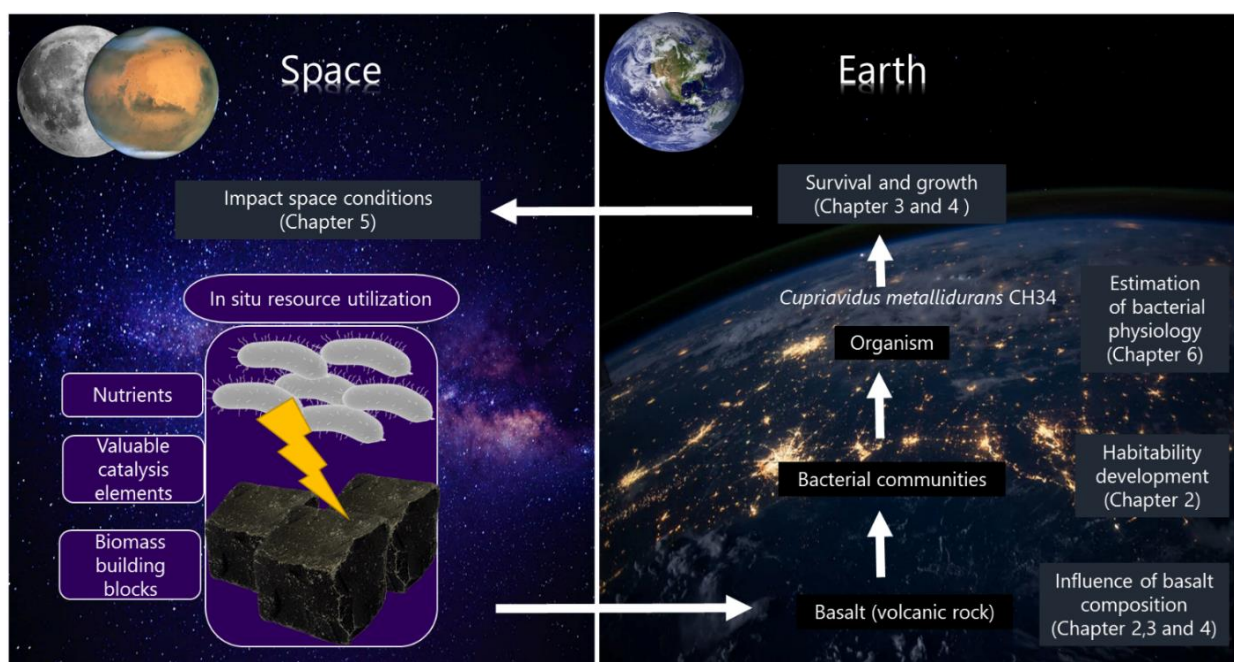


Figure 1.7: Overview of the objectives and outline of this research

Chapter 2

Bacterial communities present on
basalt deposits of different ages

Chapter 2

Bacterial communities on basalt deposits of different ages

Abstract

It was already shown that microorganisms are the primary colonizers on newly formed volcanic substrates such as lava and that subsequent microbe-mineral interactions contributed to formation of an initial ecosystem in these environments. The role of certain bacteria in transforming these extreme conditions into a habitable environment is not well characterized and poorly understood. This study therefore used both culture dependent and independent methods to (1) determine the bacterial communities present on lava deposits of different ages and (2) look at the relationship between the bacterial community characteristics, composition and age of the lava basalt.

Differences were seen both between the different flows as well as between the different sampling sites within one flow. The young and intermediate flows appeared to be dominated by *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*, which could be the first to colonize new terrestrial basalt formations through their autotrophic, photoheterotrophic and chemolithotropic abilities, while in the old flow *Firmicutes* and *Proteobacteria* appeared to dominate. Our results could indicate that especially N₂-fixers such as *Planctomycetaceae*, *Rhizobiales* *Sphingobacteriales*, *Acetobacteraceae*, *Clostridium*, *Chitinophagaceae*, and heterotrophs such as *Ralstonia*, were responsible for shift of the population to the old flow, allowing further rhizosphere and soil bacteria to colonize this environment and contribute to habitability development.

Chapter partially redrafted after: Byloos, B. Van Houdt, R. Mysara, M. Monsieurs, P. Boon, N. and Leys, N. Chronosequencing the bacterial communities on lava deposits that make them habitable. In preparation

2.1 Introduction

Igneous rocks constitute about 95% of the Earth's upper crust and the initial processes of colonization and subsequent rock weathering by microbial communities have been poorly understood. Microbes drive the cycling and sequestration of different elements such as nitrogen, carbon and other nutrients, which facilitate subsequent pioneer and plant development, impacting long-term regulation of the Earth's temperature and biosphere. This interplay between environment and biosphere has big implications for the development of the Earth's atmosphere, not only now, but also for the early Earth (Schulz et al., 2013).

Unvegetated lava deposits lack readily available nutrients and together with other limiting factors this imposes selective conditions on possible colonizing bacterial communities, e.g. nitrogen and carbon need to be supplied via the microorganisms' N₂ and CO₂ fixating activity (Staudigel and Hart, 1983; Carson et al., 2009; Kelly et al., 2010; Kelly et al., 2011; Fujimura et al., 2012; Olsson-Francis et al., 2015). These environments also constitute an extreme environment because of exposure to higher UV radiation, temperature fluctuations and desiccation, as well as limiting organic and nitrogen availability (Kelly et al., 2011; Kelly et al., 2014). In addition, solidified lava cannot retain much water, therefore, early microbial colonization depends on exogenous precipitation of nutrients, e.g. trace gasses that could also serve as a source of carbon and energy (Gomez-Alvarez et al., 2007). The best studied igneous rock formation are oceanic basalts, as about 60% of the Earth's crust consists of basalt, and microorganisms colonizing marine basalts play an important part in biogeochemical cycling of elements in marine water (Mason et al., 2009). In addition, most research for initial colonization of terrestrial rocks has focused on the role of lichens (Chen et al., 2000b), while work on bacteria and their role in volcanic rock weathering was only recently elucidated with *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* being found to be the dominant phyla (Gomez-Alvarez et al., 2007; Cockell et al., 2009; Kelly et al., 2010; Kelly et al., 2011; Fujimura et al., 2012; Kelly et al., 2014).

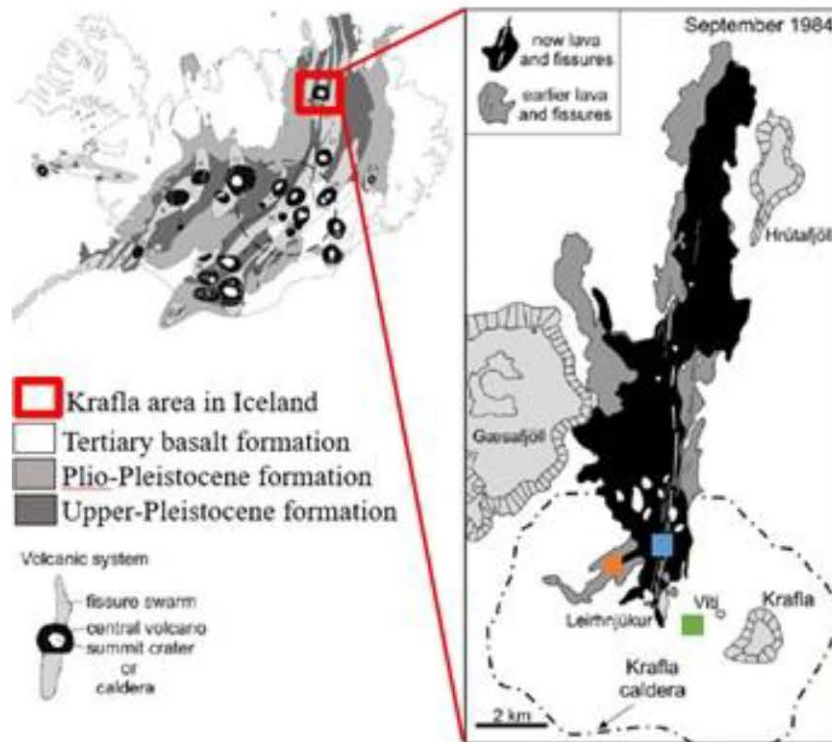
Thus, relatively few data are available on the diversity and richness of the communities inhabiting successive and chronological lava flows. As there is a constant substrate input, chronosequences are formed where older, more weathered substrates are further from the substrate source, which thus serves as a proxy for time and ecosystem

development. The latter can be used to study community shifts along this chronosequence to determine how this correlates with habitability development (Nemergut et al., 2007). In this study, we determined and compared the microbial communities of different subsequent lava deposits around the Krafla volcanic field, Iceland and determined element dynamics along the chronosequence of the lava flows.

2.2 Material and methods

2.2.1 Sample sites and sampling

Samples were taken from three different locations on the Leirhnjúkur fissures formed around Krafla, Iceland from the 1975–84 Krafla Fires. During this period, there was significant periodic rifting and faulting along the plate boundary, which was confined to a single system around Krafla. Both rifting episodes included a series of nine small ($<0.2 \text{ km}^3$) effusive eruptions that occurred on fissures within the Krafla caldera volcano as well as on the nearby sectors of the fissure swarms (Thordarson and Larsen, 2007). On 27 July 2016, 9 sample sites were defined, three on each of the three lava flows (young, intermediate and old) originating from the fissure swarms (Figure 2.1). Within each of the three flows sites A and B were in proximity to each other (10 m distance) while a third one (C) was located 100 m from sites A and B, but still within the same lava deposit. Coordinates of the young, intermediate and old lava flow positions are $65^\circ 43' 39.828''\text{N}$ - $16^\circ 47' 23.3372''\text{W}$, $65^\circ 42' 50.5332''\text{N}$ - $16^\circ 47' 26.7576''\text{W}$ and $65^\circ 42' 56.8872''\text{N}$ - $16^\circ 47' 31.3908''\text{W}$, respectively. About 20 g of smaller sized grained rocks and soil material, was sampled at 5 cm below the upper part of the deposits with an ethanol sterilized spoon and collected in a 50 ml falcon tube (Greiner Bio One, Belgium). These were sealed in 3 layers of plastic bags. Samples were then transported to SCK-CEN and prepared for culture and molecular based methods as described below.



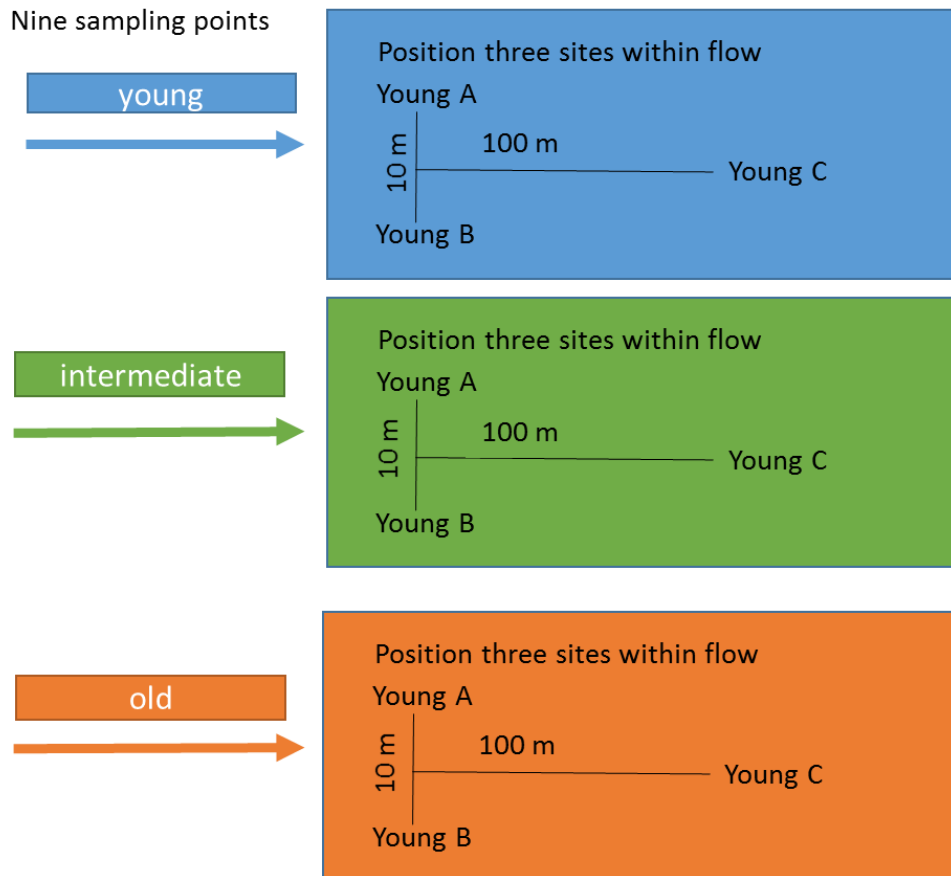


Figure 2.1: Map of the Krafla area in Iceland, location of the three different sampling locations on each of the fissures from the vent and lava flows produced by the eruptions of the 1975–84 Krafla Fires. Modified from Thordarson and Larsen (2007).

2.2.2 Culturing methods and identification of isolates

5 g of sample was added to 50 ml Butterfield's Phosphate Buffer (0.25M KH_2PO_4 in distilled water, adjusted to pH 7 with 1 M NaOH) and incubated overnight at 30°C in static conditions. Part of the buffer solution was used for total cell enumeration by Sybr Green staining and flow cytometry as described in Van Nevel et al. (2013) (see also section 3.2.3). One hundred μl of the same solution was plated on R2A medium, as a general medium for isolation. Plates were incubated at 30°C for 5 days. Afterwards the number of colonies was counted and morphologically different colonies were purified on the isolation media. For each of these purified colonies, 16S rDNA PCR amplification was performed (8R-1492R targeted rDNA primers). PCR products were purified (Wizard® SV Gel and PCR Clean-Up System, Promega) and prepared to manufacturer's protocols for sequencing (Eurofins, Germany).

2.2.3 Composition analysis

Five gram of basalt rock material, with a mesh size below 200, was prepared and used to analyze the composition of the different basalt rocks, with lithium metaborate/tetraborate fusion and ICP-OES/ICP-MS analysis, and titration for FeO (Actlabs, Canada) (SCK1-3 for the young, intermediate and old respectively in Appendix 1).

2.2.4 DNA extraction

Two different protocols were used for DNA extraction. DNA was extracted from 1 g of the basalt fraction using the bead-beating cetyl trimethylammonium bromide (CTAB) phenol extraction protocol previously described by Griffiths et al. 2000 (Griffiths et al., 2000). The second protocol is based on the FastPrep protocol from Vilchez-Vargas without the last Sepharose 4B based purification step (Vilchez-Vargas et al., 2013). Afterwards extracted DNA of each of the protocols was dissolved in 1xTE buffer and stored at -20°C.

2.2.5 Illumina sequencing

Illumina sequencing technologies have been developed to profile complex microbial systems by sequencing the hypervariable domain of the prokaryotic 16S rRNA genes. This is done through a by-synthesis based technique detecting fluorescent, reversible terminator nucleotides (Bokulich et al., 2013). Thereby several million sequences are determined on which libraries are constructed which enable the characterization of the complete microbial community in a given sample. This method, as with all PCR-based methods, is limited by a PCR primer amplification bias (Polz and Cavanaugh, 1998) as well as an DNA extraction bias (Cocolin et al., 2001) which could impact the apparent community composition, however these techniques are much more sensitive, compared to f.e. DGGE (Denaturing gradient gel electrophoresis) and allow relative quantification and comparisons among samples.

Before Illumina sequencing, the DNA concentration was quantified by the Quantifluor dsDNA sample kit on a multi-detection system (Promega, Leiden, the Netherlands). High-throughput amplicon sequencing of the V3– V4 hypervariable region (Klindworth et al., 2012) was performed with the Illumina MiSeq platform according to the manufacturer's guidelines at LGC Genomics GmbH (Berlin, Germany), generating about half a million raw paired-end reads distributed over 27 samples. Sequences were preprocessed using the OCToPUS pipeline incorporating various algorithms (Mysara

et al., 2017). First, both forward and reverse reads are quality checked via looking at k-mer frequency to identify potential false k-mers using the Hammer algorithm implemented in the SPAdes tool. Next, the contigs were created by merging the paired-end reads using the heuristic based on the difference in Phred quality scores of both reads named "make.contigs" as described in (Kozich et al., 2013) (using mothur). These contigs were later aligned to SILVA database (ref), followed by removing those having an ambiguous base, homopolymer longer than 8 nt, or a length below 400, and those incompliant with the targeted region within 16S rRNA gene using mothur "align.seqs" and "screen.seqs" commands respectively. Next, the aligned sequences were filtered and dereplicated using mothur "filter.seqs" and "unique.seqs" commands. Secondly, sequencing errors were removed using the IPED algorithm - dedicated to denoise MiSeq amplicon sequencing data (Mysara et al., 2016). Chimera were detected using the de novo mode of the CATCH algorithm (Mysara et al., 2015). Creation of the operational taxonomic units (OTUs) was performed using UPARSE ((Edgar, 2013) with default parameters (v7.0.1001_i86linux32 – commands sortbysize, cluster_otus, and usearch_global) (Pruesse et al., 2007; Schloss et al., 2009; Edgar, 2013; Kozich et al., 2013; Vilchez-Vargas et al., 2013; Mysara et al., 2015; Mysara et al., 2016). Data is available in the public repository as well as the data of the blanco ('blanco'), control sample with a pure *Cupriavidus metallidurans* CH34 culture (control_CH34) and basalt community spiked with a pure culture of *Cupriavidus metallidurans* CH34 (control_spiked).

2.2.6 Statistics

Various alpha diversity indices were used such as Chao, Shannon, and Simpson using the mothur "summary.single" command. For beta diversity, the intersection between the samples within each group (young, intermediate, and old) were visualized via venn diagrams (utilizing the mothur "venn" command) and the distances between the samples were calculated (using the mothur "dist.shared" command utilizing the Yue & Clayton measure of dissimilarity), which was later represented using the heatmap visualization (via the mothur "heatmap.sim" command), the analysis of molecular variance (via the mothur "amova" command) and non-metric multidimensional scaling (NMDS) plots (via the mothur "nmds" command). For each NMDS plot, the OTUs that show statistical significant correlation with the coordinates were indicated ($p < 0.05$, correlation $> 70\%$), see Figure 2.6.

2.3 Results

2.3.1 Site parameters and lava flow composition

The three different sampling locations represented deposits from different eruptions of the 1975–84 Krafla Fires (32, 35 and 39 years old, respectively) and showed clear progression from poorly to fully vegetated sites. At each flow, three different points were chosen, resulting in three sites (A, B and C) for the three different lava deposits (young, intermediate and old). For the Krafla area, the yearly temperature ranges from -9 to 20°C with an average of -1°C. The average annual precipitation is 21 mm (as rain- and snowfall). The young flows are still exposed to the release of volcanic SO₂ and H₂S gasses where temperatures are higher and no visible vegetation can be seen. The intermediate flow shows lichen formation while the old sampling site already shows the formation of grasses and mosses (Figure 2.1).

2.3.2 Basalt composition

The basalt composition of the different flows is similar for most elements analyzed, except FeO and Fe₂O₃ (Table 2.1).

Table 2.1: Different basalt compositions of the young, intermediate and old flow.

Minerals (wt %) [°]	Young	Intermediate	Old	Metals (ppm) ^{°°}	Young	Intermediate	Old
SiO ₂	49.41	49.15	49.42	Zn	130	130	120
Al ₂ O ₃	13.18	13.24	13.38	Cu	120	160	130
Fe ₂ O ₃	15.49	15.06	14.19	Ni	80	80	90
MnO	0.23	0.23	0.22	Cr	120	120	140
MgO	5.64	5.76	5.88	V	438	420	405
CaO	9.96	9.5	9.97	Ba	89	90	89
Na ₂ O	2.33	2.3	2.34	Sc	44	44	43
K ₂ O	0.36	0.32	0.33	La	10,3	10,1	10,6
TiO ₂	2.00	1.99	1.84	Ce	25,1	24,6	25,1
P ₂ O ₅	0.23	0,22	0.22	Nd	16	15,9	15,5
FeO	12.30	9.00	11.10	U	0,2	0,2	0,2
LOI*	0.07	2.19	1.47	Th	0,8	0,8	0,8
Total	98.9	99.96	99.25	Pb	bd**	bd**	bd**
				Nd	16	15,9	15,5
				Zr	118	120	119
				Y	30	30	29
				Sr	151	147	148
				Rb	9	7	7

*Loss on ignition

**below detection

[°] SD for all is 0.01%

^{°°} SD for all is 0.1

2.3.3 Taxonomic distribution within each flow and comparison between different flows and sites

Taxonomic profiling, on subsampled data, indicated that most of the OTUs could not be classified on family level (Figure 2.2). For the young flows, young A and B contained about 35 and 33% unclassified sequences while young C contained 58% unclassified sequences. For the intermediate flow, intermediate A and B contained 41 and 44% unclassified sequences respectively while the intermediate C site contained the most with 67% unclassified sequences. The three sites within the old flow contained 41, 52 and 50% unclassified sequences respectively. An overview of the number of raw reads, OTUs, single-read OTUs as well as OTUs contributing more than 1% to the taxonomic distribution are listed in Table 2.2. Here it can be seen that only a small fraction of all OTUs are derived from the raw reads and that major (>1%) OTUs only contribute 2 to 19% of all OTUs and taxonomic distribution of the subsampled data. Differences are also seen between the amount of OTUs present on the A, B and C sites within one flow (Figure 2.3). Here, the A and B sites share the most OTUs on the young and intermediate flows while for the old flow most OTUs belong to the B site and the amount of OTUs shared is not different between the A, B and C sites.

Table 2.2: Overview of amount of raw read, amount of OTUs, single read OTUs and OTUs with a relative abundance above 1%.

		young			intermediate			old		
		A	B	C	A	B	C	A	B	C
Classified	# reads	1673	1726	1212	1545	1451	882	1550	1247	1325
	%	65,4	67,5	47,4	60,4	56,7	34,5	60,6	48,7	51,8
	# OTUs	349	391	196	392	300	97	58	109	53
	# single-read OTUs	175	187	37	177	119	24	1	33	8
	# OTUs > 1%	11	12	4	8	7	8	10	8	10
	%	3,15	3,07	2,04	2,04	2,33	8,25	17,24	7,34	18,87
Unclassified	# reads	915	862	1376	1043	1137	1706	1038	1341	1263
	%	35,8	33,7	53,8	40,8	44,4	66,7	40,6	52,4	49,4
	# OTUs	350	340	102	418	198	55	8	98	13
	# single-read OTUs	208	197	20	224	93	6	1	41	1
	# OTUs > 1%	1	2	5	2	6	16	1	7	2

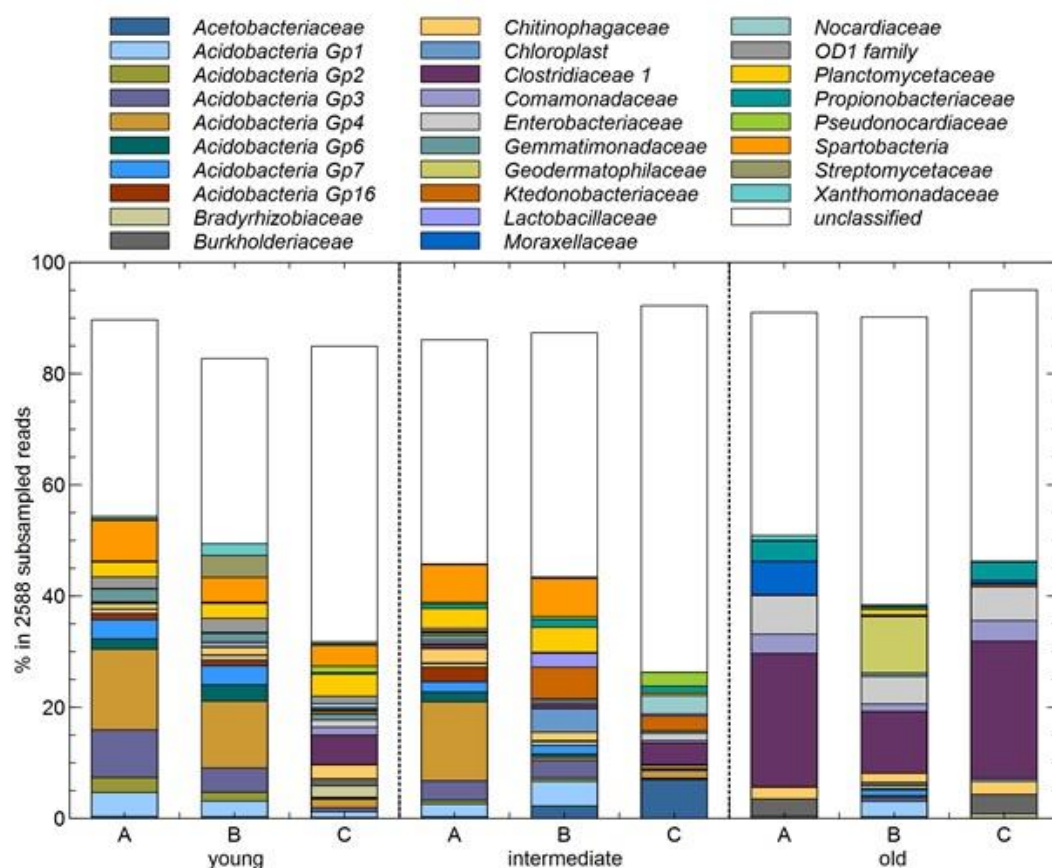


Figure 2.2: Relative abundance of the major bacterial families in young, intermediate and old flows based on 16S rRNA gene sequencing data. Only families with a relative abundance above 2% in at least one sample are shown.

The young A site covered a broad spectrum of phyla with the predominance of *Acidobacteria* (37%), *Proteobacteria* (18%), *Verrucomicrobia* (9%), *Actinobacteria* (5%) and *Bacteroidetes* (4%). The diversity in the young B site was similar to the young A site with *Acidobacteria* (29%), *Proteobacteria* (21%), *Verrucomicrobia* (6%), *Actinobacteria* (10%) and *Bacteroides* (7%). While the young C differs from the young A and B sites and is predominated by *Actinobacteria* (17%), *Proteobacteria* (12%), *Verrucomicrobia* (6%), *Firmicutes* (6%), and *Bacterioidetes* (5%). For the young A site, the two dominant phyla, *Proteobacteria* and *Acidobacteria*, the *Proteobacteria* are represented by *Sphingomonadales*, *Burkholderiales* and *Rhodospirillales* (1% each) while for *Acidobacteria* most are unclassified sequences. For the *Actinobacteria*, most are represented by *Actinomycetales* (3%), *Aciditerrimonas* (1%) and *Solirubrobacterales* (2%). Most of the *Verrucomicrobia* are unclassified while most *Bacterioides* are *Chitinophagaceae* (2%) and *Sphingobacteriaceae* (2%). For the young C site, the *Actinobacteria* are represented by *Arthrobacter* (1%), most of the *Proteobacteria* (12%) are *Rhizobiales* and *Burkholderiales*. Interestingly, *Cupriavidus*

as well as *Ralstonia* species are found within these sites (1%) (belonging to to *Burkholderiales*). The *Firmicutes* (6.5%) are represented by *Clostridiales* (5%) while for the *Verrucomicrobia* (6%) most are *Spartobacteria* (4%). In addition, for the *Bacteriodes* (4%) most are represented by *Sphingobacteriales* and unclassified *Planctomycetes* (4%).

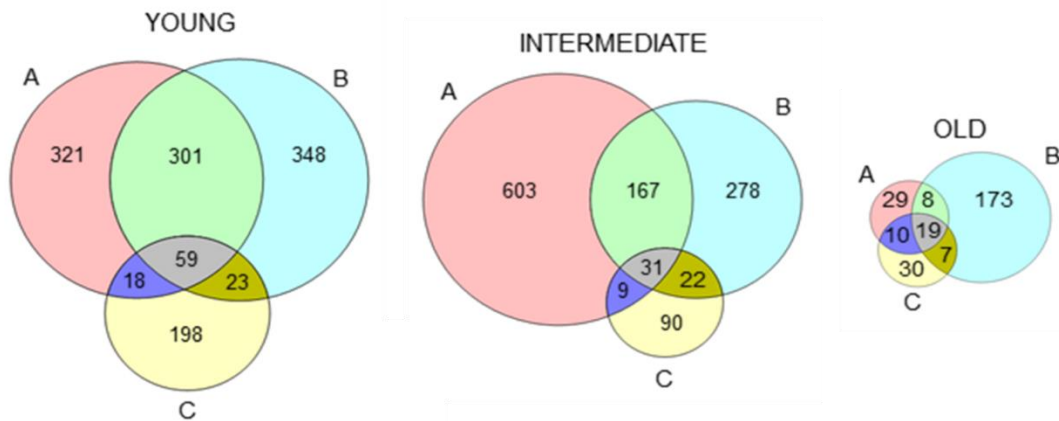


Figure 2.3: Weighted venn diagram representing the amount of OTUs present in the young, intermediate and old flow as well the amount of unique and shared OTUs for the different A, B and C sites within one flow.

When the intermediate A, B and C sites are compared, differences in distribution can be seen between all three sites. Site intermediate A is dominated by *Acidobacteria* (30%), while intermediate B is dominated by *Proteobacteria* (13%) and *Acidobacteria* (11%), intermediate C contained the most *Actinobacteria* (27%). All sites contain *Acidobacteria*, *Actinobacteria* and *Proteobacteria*, but differ in the relative abundance of these phyla. For site intermediate A, most of the *Acidobacteria* are unclassified OTUs (27%). The *Actinobacteria* phylum is represented by *Actinomycetales* (3%) and *Solirubrobacterales* (1%), most *Bacteriodes* are *Sphingobacteriales* (3%). In addition intermediate A also contains *Planctomycetales* (6%) and *Spartobacteria* (6%) For site intermediate B *Proteobacteria* are represented by *Rhizobiales* (2%) and *Rhodospirillales* (3%). Within the other dominant phylum for intermediate B (*Acidobacteria*), most sequences are unclassified. For the intermediate C site, *Actinobacteria* are represented by *Actinomycetales* (17%) and *Solirubrobacterales* (7%).

The old flow is predominated by *Firmicutes* (25%) and *Proteobacteria* (20%). No major differences were observed in their taxonomic distribution (old A, B and C). The old A site contained 30% *Firmicutes* while the other two, old B and C, contained 13% and

26%, respectively. For the old B site, about 17% were *Actinobacteria* while the other two only contained about 4%. Within the *Firmicutes* phylum both *Clostridia* (20%) as well as *Bacillus* (3%) are represented. The *Proteobacteria* phylum is represented by *Burkholderiales* (6%) (which consists of 3% *Ralstonia* and 3% *Pelomonas*), *Pseudomonadales* (3%), *Rhizobiales* (2%) (which consist of *Bradyrhizobium* (1%) and *Methylobacterium* (1%)), *Caulobacterales* (1%), *Rhodospirillales* (3%) and *Sphingomonadales* (1% *Sphingomonas* and 1% *Novosphingobium*). For the old B site, most *Actinobacteria* are unclassified.

The young, intermediate and old flows shared three OTUs classified as *Clostridium* and *Bradyrhizobium*, and an unclassified OTU (Figure 2.4). Next to the OTUs shared by all three, additional OTUs are shared when flows are pairwise compared. The young and intermediate flows shared ten OTUs belonging to *Actinobacteria* (4 OTUs), *Proteobacteria* (2 OTUs), *Acidobacteria* (1) and one unclassified OTU. Intermediate and old flows shared seven OTUs classified as *Proteus*, *Clostridium*, *Propionibacterium*, *Pelomonas*, *Morganella*, *Klebsiella* and unclassified *Bacteriodes*. The young and old flows only shared one OTU (unclassified *Proteobacteria*).

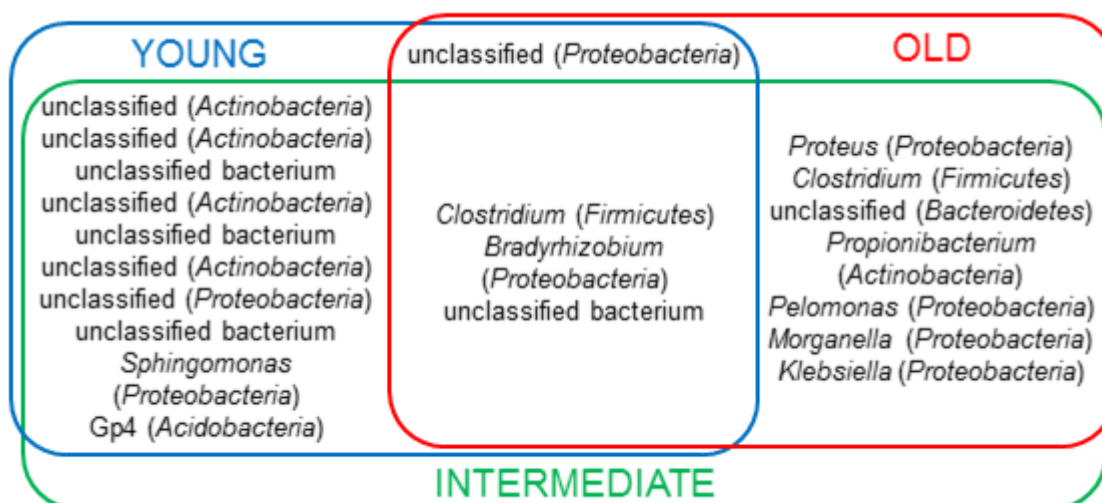


Figure 2.4: Venn diagram of the different OTUs shared between the different flows.

2.3.4 Diversity and NMDS analysis between sites and comparison

To evaluate data from next generation sequencing, ecological measurements of diversity can be determined to describe the community and its composition in a quantitative and qualitative way, indicating community structure and dynamics.

Diversity, independent of the identity of the bacteria, is defined as a measure of how much variety is present in a community (Lozupone and Knight, 2008). Many indices that have been created to measure diversity vary in a particular aspect of diversity that they measure, their sensitivity to different abundance classes and their failings. Three indices of diversity have been calculated within this work (Shannon, Simpson and Chao). The Shannon index, which is the negative sum of each species proportional abundance multiplied by the log of its proportional abundance, is a measure of the amount of information (entropy) in the system and hence of the difficulty in predicting the identity of the next individual sampled. It is equally sensitive to rare and abundant species; sensitivity to rare species increases as it decreases from 1, and sensitivity to abundant species increases as it increases from 1. Simpson's index (D) gives a strong weighting to the dominants. It gives the probability that two observations chosen at random will be from the same species. The Chao index, is a singleton based diversity analysis and will increase when more singletons (or rare OTUs) are present in the sample (Schloss and Handelsman, 2005; Jost, 2006)

Besides diversity indices, the relative abundance of each species is valuable since it describes the proportion in which each species is present in the community. Species abundance models are useful because they address the overall distribution of a sample, assisting comparison by revealing whole trends and specific changes in particular abundance classes. They are also more sophisticated to investigate diversity because they examine the distribution of abundances in a population rather than distilling all this information down into a single number (Hill et al., 2003). For beta diversity, Yue & Clayton measure of dissimilarity was calculated, which is a function of species proportions, comparing shared species proportions in each of the communities determined. With this, the more similar both species proportions are in each of the communities, the higher the weight of the index will be (Yue and Clayton, 2005).

Data was randomly subsampled to 2588 OTUs and the different alpha diversities (Simpson, Shannon and Chao1 indexes; Table 2.3) as well as beta diversity (Figure 2.5) were calculated. Homogeneity of molecular variance (HOMOVA) was performed (Table 2.3) to compare the homogeneity of the young, intermediate and old flow. In addition, rarefaction curves (Figure 2.6) were visualized to indicate if the level of subsampling adequately represents the bacterial diversity in the samples. However, this was not sufficient to fully characterize the young A and B as well as the

intermediate A and B. HOMOVA indicated that significant differences could be observed ($p = 0.022$) when comparing the young, intermediate and old flows. For alpha diversity, the different indices showed that the intermediate B site scored high for all three, while young A, B and C, and old C sites scored the lowest, indicating a diverse population with both abundant dominant species as well as the presence of unique species. Furthermore, the old A and B sites ranked higher than the intermediate B and C sites. Beta-diversity analysis indicated that the young A and B sites were most similar (>0.8), as well as the old C and A sites (Figure 2.5). Intermediate A and B also showed some similarity (0.6-0.8) with young A, B and C while intermediate C showed most similarity (0.6-0.8) with old A, B and C. Old B and C showed similarity (0.6-0.8) with each other as well as old A with old B.

Table 2.3: Overview of the different diversity indexes for each of the sampled sites and the HOMOVA analysis.

Location	Number of raw reads	Sub sampling	Observed amount of unique OTUs	Coverage	Inverse Simpson	Shannon	Chao
Young A	54256	2588	2137	0.9992	5.20	2.51	66.11
Young B	45460	2588	2101	0.9714	13.22	3.64	286.44
Young C	10620	2588	335	0.9779	13.81	4.24	323.33
Intermediate A	83250	2588	2417	0.8450	107.42	5.91	1268.29
Intermediate B	128042	2588	1322	0.9180	49.79	5.10	709.00
Intermediate C	11023	2588	175	0.9884	28.96	3.91	168.73
Old A	2588	2588	66	0.8520	85.06	5.53	1313.73
Old B	3778	2588	234	0.8516	107.97	5.68	1231.24
Old C	6852	2588	70	0.9965	3.91	2.18	72.00
HOMOVA	BValue	P-value	SS within - values				
young-intermediate - old	1,5921	0.022*	0,431115				

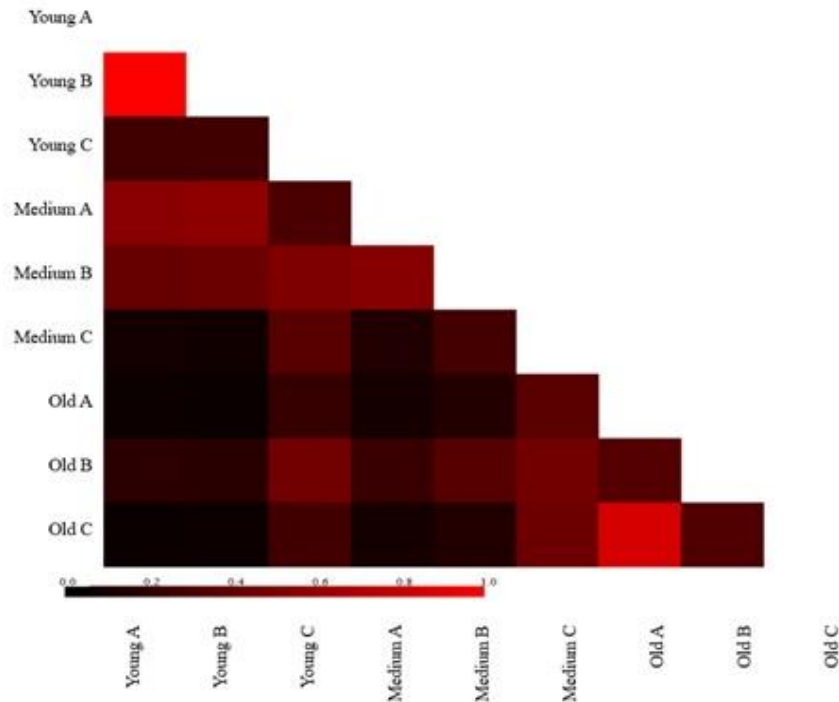


Figure 2.5: Heatmap visualization, of the Yue & Clayton measure of dissimilarity, to compare the different sites.

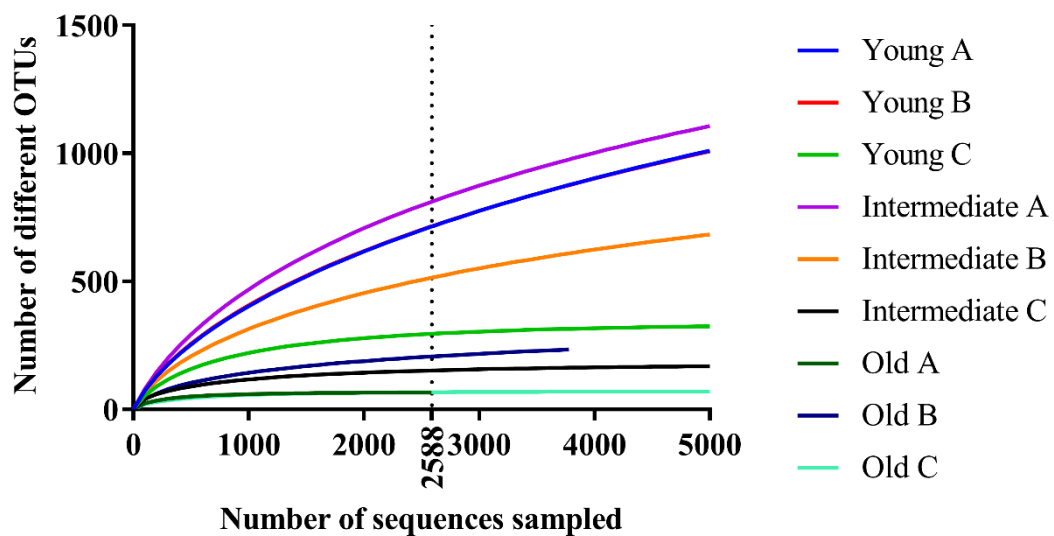


Figure 2.6: Rarefaction curves of the different samples

In addition, nonmetric multidimensional scaling (NMDS) analysis was performed to visualize the similarity of individual sampling sites and further assess the relationship between deposit age and bacterial community. The young A and B sites are similar as well as the old A, B and C sites, since these are ordinated closer together (Figure 2.7). NMDS analysis also seen with beta-diversity the Yue and Clayton measure of

dissimilarity, shows that the three A, B and C sites of the intermediate flow are spread out. The young C is situated in between the young and old cluster. This could indicate that the young C site differed from the young A and B sites and was more similar to the old flows and the intermediate B site. The intermediate samples do not cluster together, indicating more dissimilarity between all three samples. OTUs that significantly influenced the movement of the samples could now be assessed. OTUs that significantly impacted the downward movement along the Y axis responsible for the migration from the young to the old cluster were classified as OTUs corresponding to *Actinobacteria*, *Firmicutes*, *Burkholderiales*, *Sphingobacteriales*, *Solirubrobacterales*, *Rhodospirillales*, *Rhizobiales*, *Planctomycetaceae*, *Chitinophagaceae*, *Rhizobium*, *Geobacter*, *Ktedonobacteria*, *Pelomonas* and *Ralstonia*. OTUs that significantly influenced the movement along the X-axis, impacting movement between the different A, B and C sites, were classified as *Ktedonobacterales*, *Burkholderiales*, *Sphingomonadaceae*, *Verrucomicrobia*, *Spartobacteria* and *Arthrobacter*.

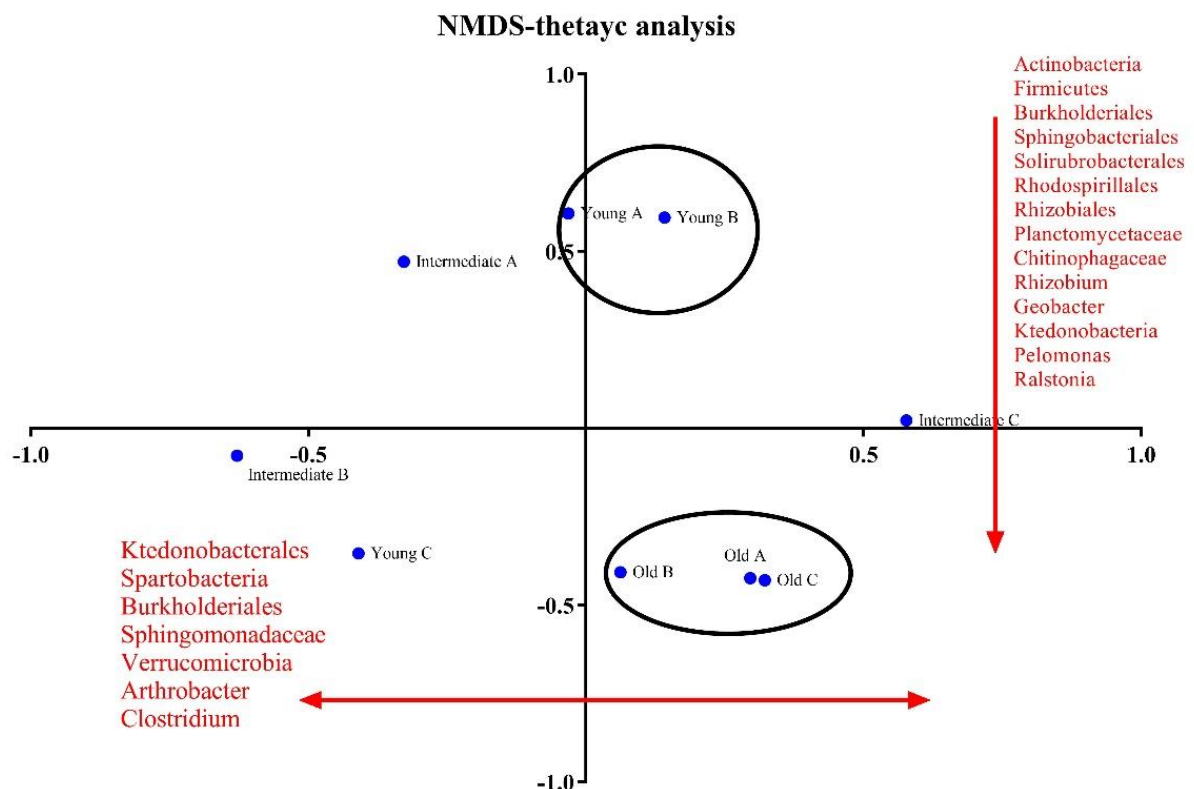


Figure 2.7: Graphical representation of the NMDS analysis

2.3.5 Isolations

The total number of cells was determined by flow cytometry counting and showed that the young flow contained 6.80×10^7 , 9.75×10^7 and 1.30×10^8 cells/g sample for the A, B and C site, respectively. For the intermediate flow, the A, B and C site contained 3.74×10^8 , 3.79×10^8 and 9.90×10^7 cells/g, respectively. The old A, B and C sites contained 6.91×10^7 , 7.54×10^8 , 9.38×10^7 cells/g, respectively (Table 2.4). Thus, all three flows contained roughly the same amount of cells. To determine the number of culturable cells in the different environments, isolations were performed on a general R2A medium. In general, viable counts were lower than total cell counts by flow cytometry (Table 2.4). Viable counts on R2A medium resulted in 1.14×10^6 , 6.00×10^6 and 2.89×10^5 CFU/gram on the young A, B and C sites respectively. For the intermediate flow this was 2.44×10^7 ; 1.61×10^7 and 3.44×10^5 respectively indicating that for the intermediate A and B this was higher than the intermediate C sample. 2.96×10^6 , 7.38×10^6 and 2.56×10^5 were counted for old A, B and C respectively similar in amount to the CFU's counted on the young flow. The young, intermediate and old C samples are similar in amount of CFU's on all flows.

Table 2.4: Overview of the results for the cultivation based methods as well as after flow cytometry analysis with Sybr Green.

CFU/gram sample on R2A	A	B	C
Young	1.14×10^6	6.00×10^6	2.89×10^5
Intermediate	2.44×10^7	1.61×10^7	3.44×10^5
Old	2.96×10^6	7.38×10^6	2.56×10^5
Cells/gram sample with Sybr Green	A	B	C
Young	6.80×10^7	9.75×10^7	1.30×10^8
Intermediate	3.74×10^8	3.79×10^8	9.90×10^7
Old	6.91×10^7	7.54×10^8	9.38×10^7

In total, 19 morphologically different colonies were isolated on R2A medium (Table 2.5). On the old B and C sites, *Pseudomonas* spp. were isolated as well as *Bacillus* sp., *Rhodococcus* sp., and *Pseudomonas* sp. From the old A site only *Bacillus* sp. as

well as *Rhodococcus* sp. were isolated. For the intermediate A site *Bacillus* sp. and *Rhodococcus* sp. were isolated, for the intermediate B site a *Pseudomonas* sp. was isolated. For the intermediate C site, next to a *Bacillus* sp. and a *Pseudomonas* sp., a *Cellulomonas* sp. was isolated. On the young flow, for the young A site *Brevibacillus*, *Burkholderia*, *Rhodococcus* sp. were isolated while for the young B site *Arthrobacter* and *Burkholderia* sp. were isolated. On the young C site only *Arthrobacter* sp. were isolated. When these 16S rRNA gene sequences were compared, after size trimming, to the OTU library, all matched with the corresponding OTU consensus sequences (identity >90%). However, none of the matched OTUs had high abundance in the taxonomic distribution.

2.4 Discussion

Little data exist on the diversity and richness of the microbial communities inhabiting successive and chronological lava flows, increasing habitability and how these microbial communities shift during subsequent lava deposits. To analyze this, samples were taken along a chronosequence, at three different locations on the Leirhnjúkur fissures formed around Krafla, Iceland, and subjected to 16S rRNA gene amplicon sequencing.

All samples contained a large fraction of unclassifiable sequences, ranging from 33% for the young B site to 67% for the intermediate C site. Similarly, Herrera et al. (2009) observed that 70% of the OTUs found in Icelandic volcanic glass were unclassifiable. Next to the fact that these environments are not well characterized, this could also be because of the presence and unspecific amplification of Archeal 18S rRNA gene sequences (Baker et al., 2003). In addition, also artifacts of DNA amplification could be present as only a low amount of DNA was extracted from all the samples. This is maybe due to the presence of DNA binding components naturally present in the sample, making DNA extraction difficult, such as humic acids from the soils within the older depositions (Lukito et al., 1998), DNA-sequestration in a hard mineral matrix making it unavailable for extraction and DNA degradation particularly due to acidic conditions present (Herrera and Cockell, 2007).

Table 2.5: Overview results cultivation based methods and characterization of the isolates after 16S rDNA based amplification and sequencing with identity sequence similarity (ISSym) and abundance matched OTU (AMO)

Isolate	Medium	Flow	Site	Phylum	Identification	ISSym	OTU match	AMO (%)
1	R2A	Old	A	<i>Firmicutes</i>	<i>Bacillus</i> sp.			
2	R2A	Old	A	<i>Actinobacteria</i>	<i>Rhodococcus</i> sp.	97%	<i>Rhodococcus</i>	0.0004
3	R2A	Old	A	<i>Actinobacteria</i>	<i>Rhodococcus</i> sp.			
4	R2A	Old	B	<i>Proteobacteria</i>	<i>Pseudomonas</i> sp.	99%	<i>Pseudomonas</i>	0.364
5	R2A	Old	C	<i>Actinobacteria</i>	<i>Rhodococcus</i> sp.	99%	<i>Rhodococcus</i>	0.0006
6	R2A	Old	C	<i>Firmicutes</i>	<i>Bacillus</i> sp.			
7	R2A	Old	C	<i>Proteobacteria</i>	<i>Pseudomonas</i> sp.			
8	R2A	Intermediate	A	<i>Actinobacteria</i>	<i>Rhodococcus</i> sp.			
9	R2A	Intermediate	A	<i>Firmicutes</i>	<i>Bacillus</i> sp.			
10	R2A	Intermediate	B	<i>Proteobacteria</i>	<i>Pseudomonas</i> sp.	99%	<i>Pseudomonas</i>	0.0008
11	R2A	Intermediate	C	<i>Firmicutes</i>	<i>Bacillus</i> sp.			
12	R2A	Intermediate	C	<i>Actinobacteria</i>	<i>Cellulomonas</i> sp.			
13	R2A	Intermediate	C	<i>Proteobacteria</i>	<i>Pseudomonas</i> sp.			
14	R2A	Young	A	<i>Firmicutes</i>	<i>Brevibacillus</i> sp.			
15	R2A	Young	A	<i>Proteobacteria</i>	<i>Burkholderia</i> sp.	99%	<i>Burkholderia</i>	0.002
16	R2A	Young	A	<i>Actinobacteria</i>	<i>Rhodococcus</i> sp.			
17	R2A	Young	B	<i>Actinobacteria</i>	<i>Arthrobacter</i> sp.	100%	<i>Arthrobacter</i>	0.063
18	R2A	Young	B	<i>Proteobacteria</i>	<i>Burkholderia</i> sp.	99%	<i>Burkholderia</i>	0.030
19	R2A	Young	C	<i>Proteobacteria</i>	<i>Arthrobacter</i> sp.			

As subsampling was too low to fully characterize the samples young A and B, and intermediate A and B, caution should be taken with the interpretation of these results. HOMOVA analysis showed significant differences between the young, intermediate and old flows, indicated that these flows significantly differ in taxonomic distribution. As seen in the NMDS plot, the different young sites seem to cluster together as well as the old sites while the different intermediate samples are more spread, possibly indicating that differences for the intermediate flow are more pronounced than for the other flows. This may indicate that especially the young flow constitute a more stringent environment to colonize, as the lowest diversity indices are seen here, allowing less variation in the community. For the intermediate and also the old flow, having higher diversity indices, it could indicate that they allow a more diverse environment and hence bacterial community opportunities (Ramette, 2007). In addition, differences are also seen between the A, B and C sites within one flow, possibly indicating spatial heterogeneity within a flow deposit of the same age, with the young and intermediate A and B sites showing more similarity than the young and intermediate C sites, while this is less seen for sites within the old flow. From NMDS analysis, *Ktedonobacterales*, *Burkholderiales*, *Sphingomonadaceae*, *Verrucomicrobia*, *Spartobacteria* and *Arthrobacter* heterotrophs were shown to be responsible for the shift in diversity between the A, B and C sites within each flow.

When the diversity of these flows is estimated with different indicators, which should be interpreted with caution, the intermediate B site shows the highest diversity followed by the old A and B sites. When compared to other basaltic environment Santelli et al. (2009) showed that seafloor basalts have a lower Chao richness ranging from 12-157, which is significantly lower than measured in all of our samples. Gomez-Alvarez et al. (2007) characterized the diversity in recent volcanic deposits, there, Chao diversity was around 70-200 and Shannon ranged from 2.6-3.87, which are the same range as these for the young flows. Kelly et al. (2014) reported lower numbers compared to our results with Chao ranging from 20-120 while the Shannon index ranged from 1.4-2.49. This could indicate that the medium and old flows sampled in this study differ from other basaltic environments and are higher in diversity, maybe due to the presence of *Proteus*, *Clostridium*, *Propionibacterium*, *Pelomonas*, *Morganella* and *Klebsiella* OTUs, which have not been associated with the basalt environment and are more abundant in the intermediate and old flow but not in the

young flow. Almost no studies have investigated the microbial communities and dynamics present in non-vegetated sections of chronosequences, except for Nemergut et al. (2007) who showed that in glacier forelands an increase in richness is correlated with increased microbial biomass and activity, which is also seen here, except for the old C site. They also showed a decrease in substrate induced respiration per unit biomass with soil age showing that these communities store carbon and that (by small subunit ribosomal RNA gene fingerprinting) there is a decrease in diversity and increase in evenness of these populations which is also seen here for the Simpson and Shannon indices from the old compared to the medium flow. This could indicate that one of the factors contributing to change within the community could thus be the age of the deposit as well the associated carbon content of the different lava flows. However, as these kind of chronosequences are not yet studied more in detail, it is difficult to draw final conclusions on the basis of these indices.

Actinobacteria, *Firmicutes*, *Burkholderiales*, *Sphingobacteriales*, *Solirubrobacterales*, *Rhodospirillales*, *Rhizobiales*, *Planctomycetaceae*, *Chitinophagaceae*, *Rhizobium*, *Geobacter*, *Ktedonobacteria*, *Pelomonas* and *Ralstonia* were indicated to impact the evolution from the young to the old flow. *Chitinophagaceae*, *Burkholderiales*, *Planctomycetaceae*, *Rhizobiales* *Sphingobacteriales* and *Acetobacteraceae* are more plant associated N₂-fixers. This shift to more N₂ fixation allows these rhizospheric bacteria to impact and colonize this environment (Teixeira et al., 2010; Mapelli et al., 2011; Knelman et al., 2012). *Rhizobiales* members have been shown to change the bacterial community structure in primary colonization of a glacier fore field (Knelman et al., 2012) as well as *Sphingobacteriales* and *Burkholderia* (*Ralstonia*) members in Arctic moraines (Mapelli et al., 2011). Thus, for the young flows, *Acidobacteria*, *Actinobacteria* and *Proteobacteria* predominated and these phyla have already been associated with the core basalt community (Gomez-Alvarez et al., 2007; Kelly et al., 2010; Olsson-Francis et al., 2015). *Actinobacteria* especially is an important phylum as members have been shown to enhance basalt weathering, resist desiccation and possess oligotrophic growth capabilities (Lechevalier and Lechevalier, 1967; Gomez-Alvarez et al., 2007; Cockell et al., 2013; Summers et al., 2013). Through their autotrophic and chemolithotrophic abilities, they could possibly act as a source of C and N in the basalt environment and leach P and S which is present (Gomez-Alvarez et al., 2007). In addition the young and intermediate flows also contain *Spartobacteria*,

which have also been isolated from the Arctic environment and other *Verrucomicrobia* were found to be associated with basalt environments where they greatly contribute to basalt weathering (Bergen et al., 2014). Intermediate and old flows contain more *Planctomycetaceae*, *Rhizobiales*, *Rhodospirillales* and *Sphingomonadales*, which have been associated with basalt communities present in different volcanic environments (Einen et al., 2006; Henri et al., 2015; Kim et al., 2015; Paul et al., 2015). In addition, *Rhizobiales* species have been associated with lichens (Erlacher et al., 2015; Oh et al., 2016). *Rhizobiales* members are also part of the community associated with deep seafloor sediments and lava formed soil (Kim et al., 2015; Lee et al., 2015). Heterotrophic bacteria have been proposed to take part in obsidian weathering (Herrera et al., 2008). The abundance of heterotrophs might indicate that they take either carbon from the rock through snowmelt or rain, or made available through the key community and use their diverse metabolic capabilities, such as heavy metal resistance, to thrive in this environment (Walsh and Clarke, 1982; Cockell et al., 2009). Especially, *Burkholderia* are a common genus in mineral rich basaltic soils and have been used to study mineral weathering, where it was shown that they release oxalate but others mechanisms could also be at play (proton-mediated dissolution, EPS production,...) (Olsson-Francis et al., 2015). *Chloroflexi* have already been associated with the seafloor basalt of Arctic ridges (Lysnes et al., 2004) and more specific *Ktedonobacter*, which were isolated from a volcanic ice cave ecosystem at Mt. Erebus, Antarctica (Tebo et al., 2015). *Sphingomonadales* have also been associated with the basalt environment impacting the alternation of basaltic glass (Einen et al., 2006). *Clostridia* are mostly associated with seafloor basalt (Lysnes et al., 2004). Others, previously not associated with basalt communities such as *Chitinophagaceae* (*Bacteroides*) were also found, which will stay latent when no cellulytic substrates are available but prevail when these substrates are available (Lauber et al., 2009; Bailey et al., 2013). Thus showing that these bacteria, after key species colonize the young flow, colonize this environment because of their oligotrophic and heterotrophic nature, by using the available C from the key species and making it easier accessible, more habitable and are able to thrive in this environment.

It has been shown that structure of bacterial communities in soil can be influenced by changes in the mineral substrate as it impacts the availability of certain element within the microhabitat (Carson et al., 2009). Differences in lava flow mineralogy may be a

contributing factor shaping microbial communities in terrestrial volcanic rocks. Rock mineralogy will affect weathering rates, nutrient availability and albedo. The latter influences the temperatures that microbes may experience within the rock. (Wolff-Boenisch et al., 2004; Hall et al., 2005; Kelly et al., 2011). Here, basalt element composition analysis showed variation in FeO and Fe₂O₃ content, which was already shown to impact and shift the bacterial communities present on basalt glass, as these iron minerals could be a source of energy (Edwards et al., 2004). The reduced iron could also become hydrated which leads to hydrogen production and could serve as electron donor for methanogens and sulfate reducers (McGrail et al., 2006; Mason et al., 2009). However, the role of this shift in iron oxide content within each of the flows, could be one of the factors contributing to the shift between the different flows. Other factors, as mentioned, such as the spatial heterogeneity, age of the deposits as well as the associated carbon content of the different lava flows and exogenous rain precipitations and wind can also impact this. In addition, factors such as porosity, pH and surface characteristics of these lava flows have also been shown to influence bacterial community composition (Barker et al., 1998; Bennett et al., 2001).

In conclusion, we used both culture dependent and independent methods to determine the composition and evolution of pioneer microbial communities colonizing subsequent lava flows at the Krafla, Iceland. Differences were seen between the different flows as well as between the different sampling points within one flow; with a large part of unclassifiable OTUs. The young and intermediate flows appeared to be dominated by *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*, which could be the first to colonize new terrestrial basalt formations through their autotrophic, photoheterotrophic and chemolithotrophic abilities, while in the old flow *Firmicutes* and *Proteobacteria* appeared to dominate. Both *Acidobacteria* and *Actinobacteria* have already been shown to input C and N into the basalt environment as well as leach P and S which is present, through their autotrophic and chemolithotrophic abilities. The presence of heterotrophs might indicate that they thrive on the C and N provided by the previous communities while they use their diverse metabolic capabilities and adaptation to thrive in this oligotrophic environment, potentially making it more habitable. The basalt environment seems thus, to be first colonized by specialist species as it constitutes a more extreme environment as it undergoes periodic desiccation, higher solar irradiation and snow cover. Our results could indicate that

especially N₂-fixers such as *Planctomycetaceae*, *Rhizobiales* *Sphingobacteriales*, *Acetobacteraceae*, *Clostridium*, *Chitinophagaceae*, and heterotrophs such as *Ralstonia*, were responsible for shift of the population to the old flow, allowing further rhizosphere and soil bacteria to colonize this environment and contribute to habitability development and evolve to a *Firmicutes* and *Proteobacteria* predominated community which is associated with lichen and plant development.

2.5 Acknowledgements

This work was supported by the European Space Agency (ESA-PRODEX), Belgian Science Policy (BELSPO) through the E-GEM/BioRock project (Bo Byloos) and the Inter-University Attraction Pole (IUAP) “μ-manager” funded by the Belgian Science Policy (BE, 305 P7/25). We thank Kai Finster and Charles Cockell for the coordination of the BioRock project.

Chapter 3

Growth in the presence of basalt

Growth in the presence of basalt

Abstract

It is well established that mineral weathering and the subsequent release of chemical elements results from a combination of abiotic and biotic processes, impacting the geochemical cycling of elements and CO₂ fixation. Since crystalline rocks of volcanic origin have complex mineral compositions, each of these minerals behaves differently when exposed to weathering. However, the influence of these different rock types and composition on element leaching and bio-weathering processes is poorly understood. Here, the role of basalt composition on nutrient leaching (calcium, iron, phosphorus or magnesium) and subsequent support of *Cupriavidus metallidurans* CH34 growth was evaluated. Seven different basalts were examined, each leaching different amounts of calcium, iron, magnesium and phosphorus. In iron limiting media, *C. metallidurans* growth could be restored for all basalt tested, while only two basalts could restore the growth in magnesium-limiting medium. Lack of phosphorus could not be complemented by any of the basalts tested. Furthermore, more subtle differences were seen as growth rates were different in the presence of the various basalts tested. Significant alterations were observed in the final cell density reached as well as in the duration of the lag phase. Except for magnesium, complementation was not correlated with the elemental composition of the basalt. Thus, different basalt compositions leach different amounts of elements, which are not correlated with rock composition, affecting element availability and bacterial growth.

Chapter partially redrafted after: Byloos, B. Harsh, M. Van Houdt, R. Boon, N. and Leys, N. The ability of basalt to leach nutrients and support growth of *Cupriavidus metallidurans* CH34 depends on basalt composition and element release. Accepted in Geomicrobiology

3.1 Introduction

Abiotic or chemical weathering of crustal rocks is one of the principal processes controlling the geochemical cycling of elements at the Earth's surface. Chemical erosion of rocks consumes atmospheric carbon dioxide and leaches metals (Garrels and Mackenzie, 1971). Basalts are among the most easily weathered rocks compared to other crystalline silicate rocks. Louvat and Allègre (1997) showed that basalt weathering acts as a major atmospheric CO₂ sink (Dupré et al., 2003). Dessert et al. (2003) estimated the CO₂ flux consumed by chemical weathering of basalts to be about 4.08×10^{12} mol/year, representing 30% to 35% of the total flux derived from continental silicate weathering (Gaillardet et al., 1997; Dupré et al., 2003). As basalt and bio-alternation of basaltic glass also served as a substrate for early Earth, these could also be important for understanding how life developed (Staudigel et al., 2008; Schulz et al., 2013).

It is well established that mineral weathering results from a combination of abiotic and biotic processes that contribute to the release of chemical elements entrapped in their crystal structure (Staudigel et al., 2008). On the one hand, microorganisms can impact and accelerate the release of certain scarcely available elements, such as iron, phosphate,..., via different mechanisms, including pH modification of the surrounding solution and production of chelating molecules as well as directly changing mineral saturation (Welch and Banfield, 2002; Wu et al., 2007; Cockell et al., 2009; Dong, 2010; Kelly et al., 2016). On the other hand, microbial communities in terrestrial volcanic rocks are shaped by rock mineralogy, which influences weathering rates, nutrient availability and microhabitat properties such as pore size and temperature (through rock albedo) (Wolff-Boenisch et al., 2004; Hall et al., 2005; Kelly et al., 2011; Kelly et al., 2016). For instance, Icelandic bacterial communities were found to differ on volcanic rocks of different chemical composition (Kelly et al., 2010).

Crystalline rocks of volcanic origin have complex mineral compositions and each of these minerals behaves differently when exposed to weathering (Olsson-Francis et al., 2012). However, the influence of different rock types and compositions on element leaching and bio-weathering processes is poorly understood, therefore, we aimed in a first stage to evaluate elemental release from seven different basalts with different composition was characterized and the influence on growth and physiology of

C. metallidurans CH34 was determined in a mineral medium deficient in calcium, iron, phosphorus or magnesium.

3.2 Material and methods

3.2.1 Strain and media composition

Cupriavidus metallidurans type strain CH34 was routinely cultured at 30°C in Tris buffered mineral (284MM) medium supplemented with 2 g/l sodium gluconate (Merck, VWR) as sole carbon source, with following composition: (final concentrations in the medium) 50 mM Tris base, adjusted to pH 7.0 (Sigma), 80 mM NaCl (VWR), 20 mM KCl (VWR), 20 mM NH₄Cl (VWR), 3 mM Na₂SO₄ (VWR), 1 mM MgCl₂·6H₂O (VWR), 0.2 mM CaCl₂·2H₂O (VWR), 0.004% Na₂HPO₄·2H₂O (VWR), 0.00025% FeCl₃ (VWR), and 0.2% Na-gluconate (VWR). A trace element solution was added from a 1000x stock solution, with following composition: (final concentration in the medium) 0.025% HCl 10 µM (VWR), 0.5 µM ZnSO₄·7H₂O (VWR), 0.5 µM MnCl₂·4H₂O (VWR), 1 µM H₃BO₃ (Sigma), 0.8 µM CoCl₂·6H₂O (VWR), 0.1 µM CuCl₂·2H₂O (VWR), 0.1 µM NiCl₂·6H₂O (VWR), 0.15 µM Na₂MoO₄·2H₂O (VWR) (Mergeay et al., 1985). Liquid cultures were grown in the dark on a rotary shaker at 150 rpm, for culturing on agar plates 2% agar (Thermo Scientific, Oxoid) was added.

3.2.2 Growth setup and analysis

Three independent cultures of *C. metallidurans* CH34 were grown to stationary phase (OD_{600nm} ~ 1), cells were harvested and washed three times with 10 mM MgSO₄. Ten µL of this cell suspension was diluted 1/1000 in cell culture flasks (Cellstar, Greiner Bio One, Germany) containing 10 ml of 284MM, 284MM without calcium (284MM-Ca), 284MM without magnesium (284MM-Mg), 284MM without iron (284MM-Fe) and 284MM with low phosphorus (30 µM) concentration (284MM-P). Each medium was used with and without the addition of different basalt types. Cultures supplemented with basalt contained 10 % (w/v) of crushed basalt (1-2 mm in size) that was washed in deionized water and heat sterilized beforehand. Flasks were incubated in static conditions at 30°C and growth was recorded at 0, 18, 24, 42, 48, 72 and 92 hours via aspiration of 300 µl of the medium of which (1) 200 µl was used to measure optical density (OD_{600 nm}) (Clariostar, BMG LABTECH) and to estimate the number of viable cells by plating a serial dilution on 284MM agar and (2) 10 µl for flow cytometry analysis (see below). The remaining ±7 ml was used for ICP-OES analysis after 92 hours (see section 3.2.4 elemental analysis). A control without cells, containing only 284MM

medium and basalt, was also prepared to determine the amount of elements leached out from the different basalt types.

3.2.3 Flow cytometry analysis

Flow cytometry is a rapid and easy technique to characterize suspended particles such as bacteria in solution and can be used to study bacterial physiology. Through hydrodynamic focusing, single cells will travel through a light beam while the scattered light is detected. This scattering can give an indication of cell size, density and morphology. In addition, by using different fluorescent dyes for specific bacterial morphological, structural or functional cell properties such as cell integrity, membrane potential and cell activity, different cell fractions can be quantified through their detection in different fluorescent channels (Nebe-von-Caron and Mueller, 2007).

Within this chapter a double staining is used to distinguish intact and permeabilized cell fractions as well as to determine total cell numbers. Therefore, samples were stained with SYBR Green (SG) to detect total and intact cell populations (Veal et al., 2000; Berney et al., 2007; Berney et al., 2008), as well as stained with propidium iodide (PI) to determine the number of permeabilized cells (Berney et al., 2007). This was done according to the optimized procedures described in Buysschaert et al. (2016), Van Nevel et al. (2013) and SLMB (2012). Samples were prepared by diluting 10 μ l of the cell suspensions 10,000 times in 0.2 μ m filtered Evian mineral water, as this gave the lowest background fluorescence. Next, SGPI dye (SYBR Green (Sigma Aldrich; final concentration of 1x) and PI (Sigma Aldrich; final concentration of 1 μ M)) was added and cell suspensions were incubated at 35°C for 13 min. Work solutions of SGPI were prepared in DMSO and kept at 4°C. These were prepared from stock solutions in DMSO kept at -20°C.

Stained bacterial suspensions were analyzed on an Accuri C6 (BD, Erembodegem) with a blue (488 nm, 20mW) and red (640 nm, 14.7 mW) laser which was calibrated according to the manufacturer's recommendation. Standard optical filters were used and included FL-1 (530/30 nm) and FL-3 (670 LP) for the blue laser. SYBR Green was detected with FL-1, PI was detected on FL-3. A quality control with 6- and 8-peak fluorescent beads (by manufacturer BD, Erembodegem) and cleaning cycle was performed prior to experiments to assess both the accuracy (bead count and position) and the cleanliness of the machine. Samples were analyzed using the Accuri C6

software (version 1.0.264.21). The flow files were deposited in the FlowRepository database with identifier FR-FCM-ZY78.

3.2.4 Elemental analysis

Inorganic element concentrations in the liquid media were measured at the end of the growth experiment. The cell suspensions were centrifuged (10 000 x g; 15 min) to pellet the cells. Next, the supernatant was filtered through a 0.22 µm filter, samples were diluted to obtain a total dissolved solid content of 0.05%, and 70% nitric acid was added. Samples were prepared in duplicate, one sample was used to determine the final inorganic element concentrations by ICP-OES and one for phosphate (PO_4^{3-}) ion chromatography (both performed by Actlabs, Canada). In addition, iron and copper values were all below detection limit.

3.2.5 Basalt composition

Five gram of basalt rock material, with a mesh size below 200, was prepared and used to analyze the composition of the different basalt rocks, with lithium metaborate/tetraborate fusion and ICP-OES/ICP-MS analysis, and titration for FeO (Actlabs, Canada). Single elements are detected and are reported as oxides, however, as mineralogy was not performed, the occurrence of these elements within the different minerals was not determined. Seven different basalt rocks were characterized and used in this study: four from Icelandic origin (SCK1-4), one from Hawaii (SCK5), one from the Eifel area (Germany) (SCK6) and one from Norway (SCK7). Their SiO_2 content was 49.41%, 49.15%, 49.42%, 51.1%, 51.51%, 47.41% and 48.78%, respectively. Their alkali content ($\text{Na}_2\text{O}+\text{K}_2\text{O}$) was 2.69%, 2.62%, 2.67%, 1.81%, 2.69%, 2.75% and 2.57%, respectively, classifying them as basalt according to the TAS diagram (Stroncik and Schmincke, 2001) (Figure 3.1 and Appendix 1).

3.2.6 Statistical analysis

For the statistical analysis of the data the GraphPad Prism (version 7.0) software package was used. For all the flow cytometry data, normal distribution was assumed, while homogeneity of variances was tested with Levene's test and a two-tailed, one way ANOVA was used with Tukey post-hoc testing ($\alpha = 0.05$). For growth fitting, DMFit Excel add-in was used (Baranyi and Roberts, 1994) to fit the OD data and one way ANOVA was used with Tukey post-hoc testing ($\alpha=0.05$) to compare the different growth parameters.

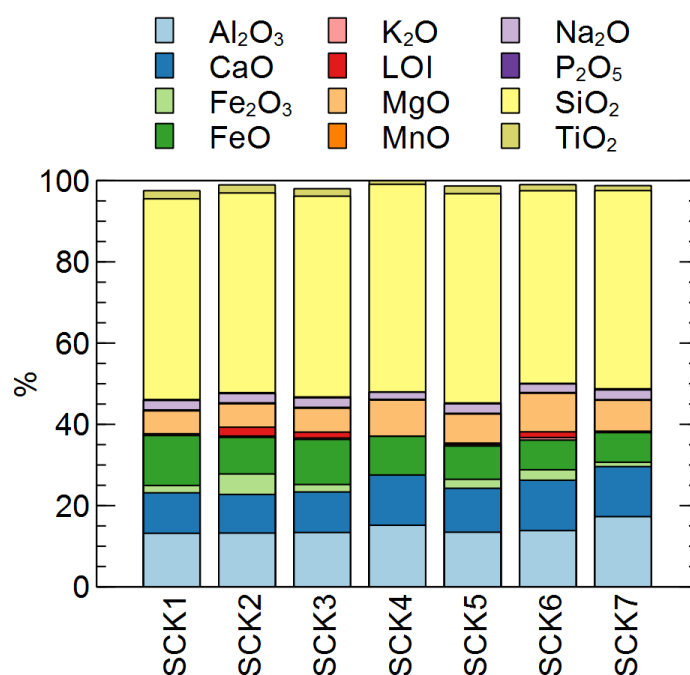


Figure 3.1. Composition of the different basalts tested.

3.3 Results

3.3.1 Growth kinetics

To test the ability of different basalt compositions to support the growth of *C. metallidurans* CH34, the effect of elemental deficiency on growth was analyzed first (Figure 3.2). Four inorganic elements were tested; calcium, magnesium, iron and phosphorus. Growth of *C. metallidurans* CH34 is affected by magnesium, iron and phosphorus deficiency, resulting in a decreased growth rate and lower final cell density ($p=0.0001$). In contrast, calcium deficiency had no effect on growth.

Next, the ability of different basalt compositions to leach particular elements and allow growth restoration was evaluated. Seven different rocks were used in this study: four from Icelandic origin (SCK1-4), one from Hawaii (SCK5), one from the Eifel area in Germany (SCK6) and one from Norway (SCK7). Growth kinetic parameters (lag phase, growth rate (k), start (Y_0) and final OD (Y_m)) were determined (Table 3.1) to evaluate the impact of different basalt compositions.

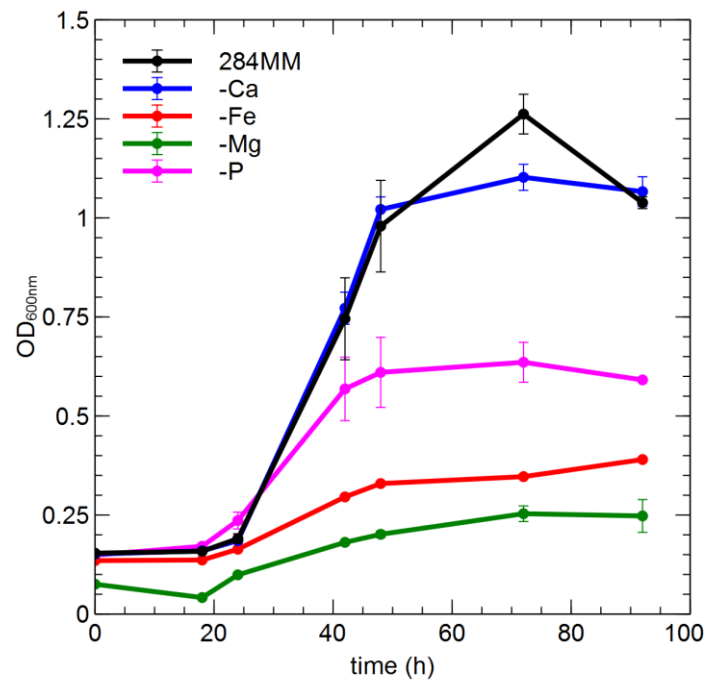


Figure 3.2. Growth of *C. metallidurans* CH34 in 284MM (black) and 284MM without calcium (blue), phosphate (pink), iron (red) and magnesium (green). Each time point is represented as a dot of the mean with SD ($\alpha = 0.05$).

As a first control, the effect of basalt addition to complete 284MM was tested, indicating that none of the basalts tested exerted hindrance of growth in complete 284MM (Figure 3.3). When modelled data was compared, slight differences could be observed as growth rates were significantly lower in 284MM with SCK1 and SCK3 ($p=0.04$), while it was significantly higher in 284MM with SCK4 ($p=0.0065$). This was also seen for the lag phases, as these were significantly shorter for cells grown in 284MM with SCK6 ($p=0.0002$). Growth in complete 284MM with SCK1, SCK5 and SCK6 resulted in lower final cell densities ($p=0.05$; $p=0.002$ and $p=0.0001$, respectively), however these differences are not seen for the absolute values in the curves.

Growth in 284MM-Ca was similar to that in 284MM-Ca with basalt (irrespective of the different compositions) and complete 284MM (Figure 3.3). No significant differences were seen for the different growth parameters modeled.

In 284MM without iron (284MM-Fe), growth of *C. metallidurans* CH34 is fully restored for all basalts (Figure 3.3), which resulted in growth parameters similar to those in 284MM. Modelled Y_m in 284MM-Fe with the different basalts are however significantly lower than those in 284MM although this difference is not seen in the curves.

In 284MM with a low phosphorus concentration (284MM-P) growth of CH34 was not restored by the addition of basalt (Figure 3.3). Modelled parameters were thus all significantly lower than measured in 284MM ($p < 0.02$). In the presence of basalt SCK6 and SCK7, Y_m was higher compared with 284MM-P ($p = 0.02$ and 0.002 respectively) but not with the others.

In 284MM without magnesium (284MM-Mg) growth is partially restored for basalt SCK1, SCK2, SCK3, SCK5 and SCK7, and fully restored for SCK4 and SCK6 (Figure 3.3). For all basalt compositions tested, except SCK7, Y_m was thus significantly higher compared to Y_m in 284MM-Mg. Only in the presence of basalt SCK4 and SCK6, Y_m was comparable to that measured in 284MM full medium, while others are significantly lower ($p = 0.0001$). In addition, growth rates were significantly lower in 284MM-Mg medium with SCK1, 3, 5 and 7 ($p = 0.02$) while growth rates with SCK2, 4 and 6 were comparable to that seen in 284MM full medium although no hindrance can be seen on the curves.

3.3.2 Effect of basalt on total cell fractions and physiology

Flow cytometry was used to evaluate the amount of total, intact and permeable cells during growth (Figure 3.4). In 284MM-Fe, 284MM-Mg, and 284MM-P the number of total and intact cells in stationary phase are one log lower compared to 284MM, which is similar to that seen with OD measurements.

Next, the different basalts were added to the different limiting media. Addition of basalt to 284MM and 284MM-Ca showed no effects, indicating that the different basalt compositions did not impact growth (Figure 3.4). When final intact cell concentrations were compared, significantly more intact cells were present in 284MM with SCK3 while it was lower with SCK1 and 6 ($p = 0.01$ for all), which corresponds to that seen for the OD data. Final concentrations were also higher in 284MM-Ca with SCK3 ($p = 0.007$) compared to 284MM, which was not seen with OD data.

When the different basalts are added to 284MM-Fe, growth is comparable to 284MM (Figure 3.4). Thus, final intact cell concentrations are significantly higher in 284MM-Fe in the presence of basalt compared to 284MM-Fe ($p = 0.0001$ for all). Final cell densities are also significantly higher in 284MM-Fe with SCK3 ($p = 0.0001$) compared to 284MM.

For phosphorus, addition of any of the basalts tested did not restore growth and was comparable to growth with low phosphorus (Figure 3.4). Final intact cell concentrations

in 284MM with SCK2 and SCK3 were significantly higher than those reached in 284MM-P ($p=0.03$ and $p=0.0002$ respectively), but significantly lower than measured in 284MM ($p=0.0001$). This is not seen for the OD data.

For magnesium, growth in 284MM-Mg with SCK4 and SCK6 was comparable to that in 284MM (Figure 3.4). In contrast with the OD measurements, intact cell concentrations in 284MM-Mg with SCK1 and SCK7 reached the same level at 92h as in 284MM, while intact cell numbers are significantly lower before 92 hours compared to 284MM and their growth curves are thus different than those seen for 284MM.

Next, in 284MM-Mg final intact cell concentrations with SCK2, SCK3 and SCK5 were significantly higher compared to 284MM-Mg ($p=0.0004$) but lower than 284MM ($p=0.0001$) (Figure 3.4).

Next, the amount of permeabilized cells was also evaluated (Figure 3.4). No significant differences were observed between the different basalt tested and the limiting media. Curves of the permeable population are significantly different when compared to the curves obtained for the amount of total and intact cells, showing that during growth, part of the population stays more permeant for PI entry, indicating a shift in cell membrane composition. This contributed not more than 10% of the total cell population in all the conditions tested.

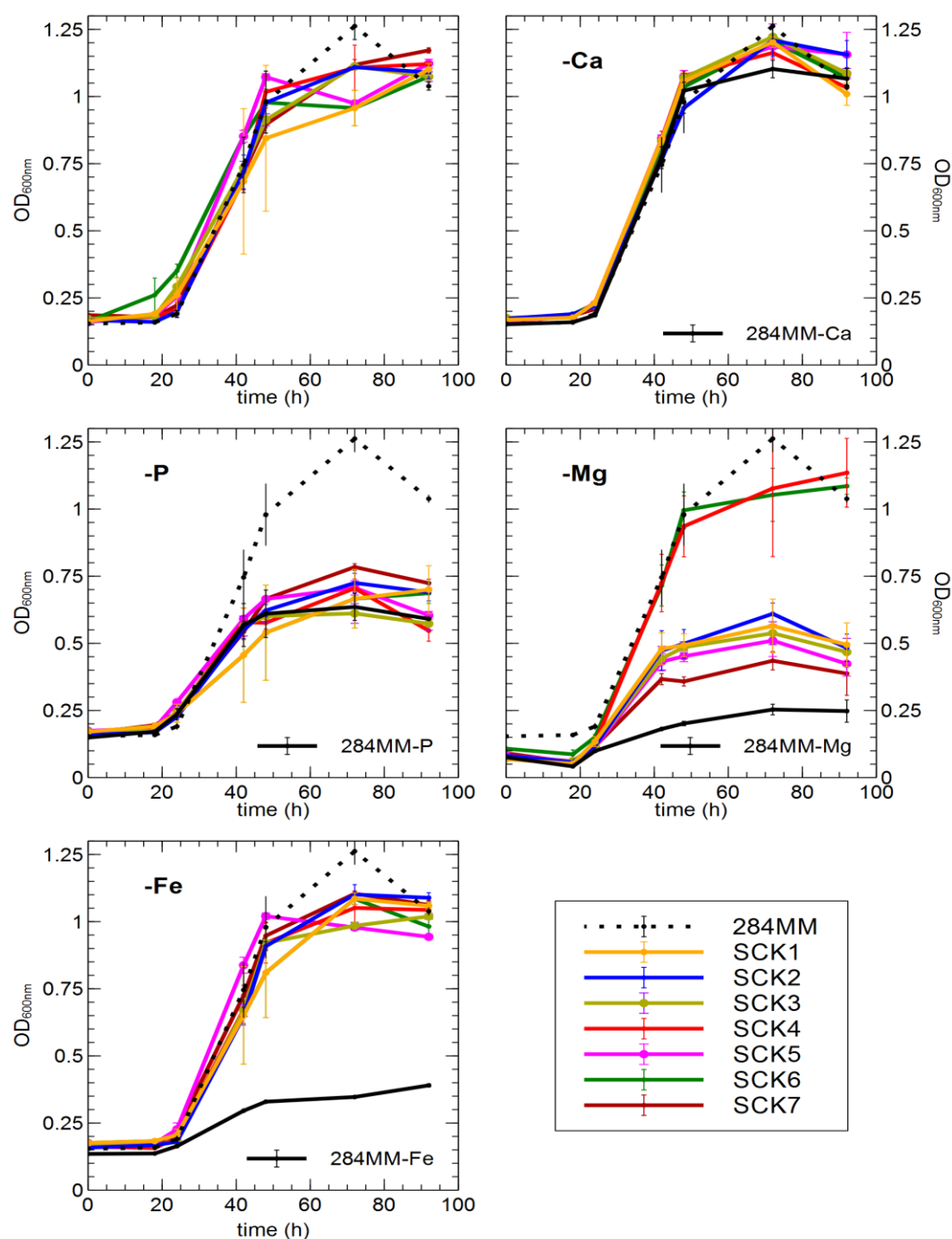


Figure 3.3. The effect of basalt type on the growth of *C. metallidurans* CH34 in 284MM (top left) and 284MM deficient in a calcium (-Ca), iron (-Fe), phosphate ($-\text{PO}_4^{3-}$) and magnesium (-Mg). Growth in 284MM (black full line) and 284MM deficient in a particular element (black dotted line) without the addition of basalt are included in each graph. Each time point is represented as a dot of the mean with SD ($\alpha=0.05$).

Table 3.1: Summary of the different growth kinetics (end OD (Y_M); start OD (Y_0) and growth rate (k)) in the full medium and medium without certain elements (magnesium (Mg), iron (Fe) phosphate (PO_4^{3-}) and calcium (Ca)) and without (284MM) and supplemented with different basalt types. Numbers in bold are significantly different ($p < 0.05$) from the values determined in 284MM full medium without basalt, while numbers underlined are significantly different ($p < 0.05$) from the values determined in 284 MM deficient medium. Red and green indicate if this is higher (green) or lower (red) than measured in 284MM full medium without basalt.

complete	284MM	SCK1	SCK2	SCK3	SCK4	SCK5	SCK6	SCK7
k (/hour)	0.042	0.027	0.047	0.028	0.061	0.043	0.030	0.033
lag	28.127	22.256	29.714	21.401	33.682	25.176	18.443	25.740
Y_0	0.157	0.157	0.167	0.163	0.183	0.176	0.175	0.176
Y_M	1.150	1.045	1.099	1.094	1.114	1.061	1.017	1.145
min Ca								
k (/hour)	0.056	0.043	0.036	0.046	0.058	0.114	0.041	0.050
lag	30.741	26.271	25.677	27.536	29.408	30.143	26.233	29.157
Y_0	0.163	0.175	0.172	0.183	0.185	0.175	0.178	0.180
Y_M	1.085	1.108	<u>1.183</u>	1.155	1.101	<u>1.172</u>	1.138	1.144
min Fe								
k (/hour)	0.008	0.033	0.045	0.048	0.046	<u>0.110</u>	0.033	0.037
lag	21.177	27.849	<u>31.320</u>	<u>31.536</u>	<u>30.928</u>	30.135	27.922	26.677
Y_0	0.131	0.173	0.166	0.170	0.169	0.181	0.173	0.168
Y_M	0.368	<u>1.072</u>	<u>1.095</u>	<u>1.002</u>	<u>1.047</u>	<u>0.981</u>	<u>1.033</u>	<u>1.084</u>
min Mg								
k (/hour)	0.002	0.021	0.066	0.020	0.042	0.021	0.056	0.011
lag	20.811	21.131	<u>23.232</u>	22.616	<u>26.175</u>	<u>23.286</u>	31.205	21.822
Y_0	0.054	0.054	0.065	0.064	0.068	0.070	0.113	0.074
Y_M	0.251	<u>0.522</u>	<u>0.546</u>	<u>0.500</u>	<u>1.112</u>	<u>0.463</u>	<u>1.070</u>	<u>0.408</u>
min PO_4^{3-}								
k (/hour)	0.021	0.015	0.019	0.021	0.025	0.020	0.014	0.020
lag	20.154	24.367	21.185	20.898	20.937	19.164	22.635	22.827
Y_0	0.150	0.171	0.154	0.167	0.162	0.167	0.169	0.174
Y_M	0.620	0.693	0.706	0.596	0.618	0.661	<u>0.718</u>	<u>0.754</u>

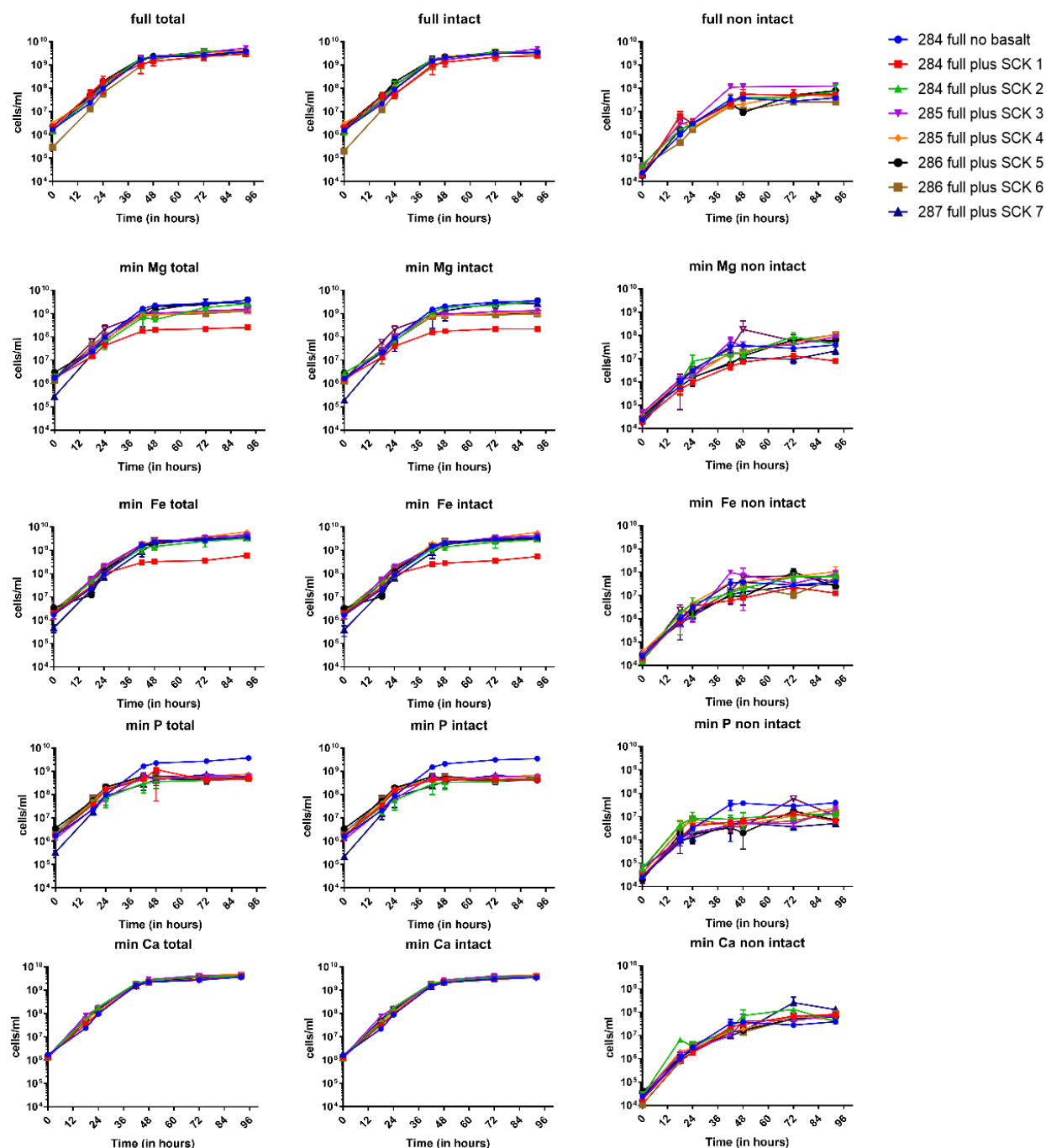


Figure 3.4: Growth followed up with flow cytometry and SGPI staining allowing to determine the amount of total cells (left), intact cells (middle) and more permeant cells (right) in 284MM medium with and without different types of basalt and with and without certain elements (magnesium (Mg), iron (Fe) phosphate (PO_4^{3-}) and calcium (Ca)). Each time point is represented as a dot of the mean with SD ($\alpha=0.05$).

3.3.3 Effect of basalt composition on element release

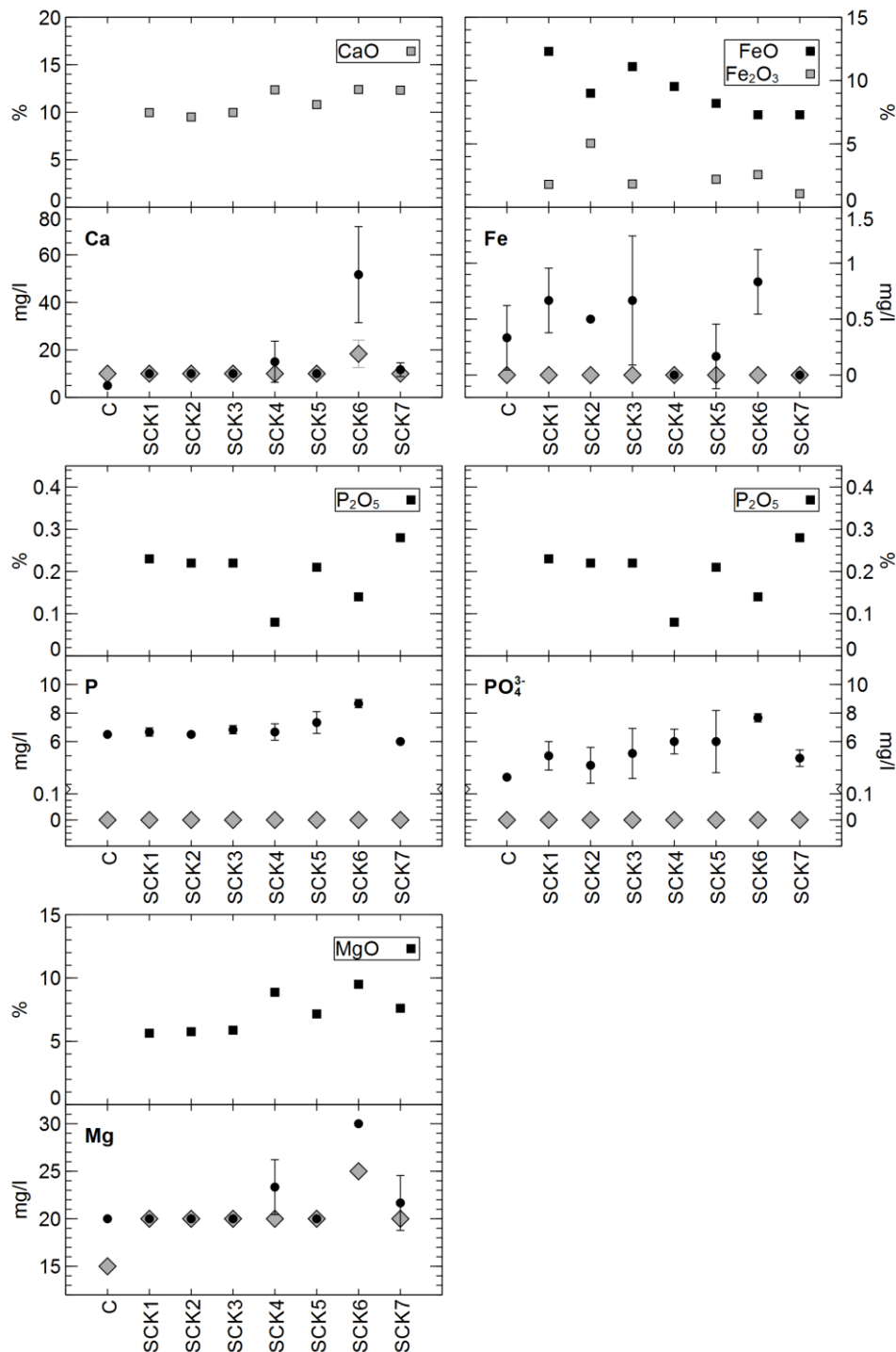


Figure 3.5.: Basalt composition (calcium oxide (CaO), iron oxide (FeO), ferric oxide (Fe₂O₃), phosphorus oxide (P₂O₅) and magnesium oxide (MgO), of the different basalt types (upper graphs) and element analysis (ICP-OES) of calcium (Ca), iron (Fe), phosphorus (P), phosphate (PO₄²⁻) and magnesium (Mg) in 284MM supplemented with the different basalt types and deficient in the element to be analyzed (lower graphs). Analyses were performed after 92h with (grey diamond) or without (black circle) *C. metallidurans* cells. As a control (C), element analysis of 284MM with *C. metallidurans* cells at time 0h (black circle) and 92h (grey diamond) was included. Each measurement is represented as a dot of the mean with SD (alpha= 0.05).

Elemental analysis was performed to evaluate elemental release from the different basalts and uptake by *C. metallidurans* CH34. Consumption of 284MM by CH34 showed that iron, phosphorus and magnesium are limiting factors, while calcium is not (Figure 3.5), which corresponded to the OD and flow cytometry measurements (Figure 3.3 and 3.4 respectively). The addition of basalt to the medium showed that leaching depended on the element as well as the basalt composition (Figure 3.5).

For calcium, each basalt tested increased the concentration in 284MM-Ca to levels comparable with basalt-free complete 284MM or higher (for SCK4 and SCK6). The amount leached was not correlated with the basalt composition.

For iron, basalt SCK1, SCK2, SCK3 and SCK6 increased the concentration, whereas basalt SCK4, SCK5 and SCK6 decreased the concentration in 284MM-Fe compared with basalt-free complete 284MM. Nevertheless, growth of CH34 in 284MM-Fe was fully restored for all basalts (Figure 3.3 and 3.4). The amount leached was not correlated with the basalt composition.

For phosphate and phosphorus, all basalts tested could supply this in levels higher or comparable to those in 284MM (Figure 3.5). However, growth was not restored (Figure 3.3 and 3.4). Again, these observations did not correlate with basalt composition.

For magnesium, the presence of each of the basalts tested, increased the concentration in 284MM-Mg to levels comparable with basalt-free complete 284MM or higher (for SCK4, SCK6 and SCK7). Although magnesium levels increased at least to those observed in basalt-free complete 284MM and concentrations in CH34 spent medium were higher than those in spent basalt-free complete 284MM, growth of CH34 was only fully restored for basalt SCK4 and SCK6. The amount leached correlated with the basalt composition (Figure 3.5).

3.4 Discussion

To determine the influence of basalt composition on element leaching and bio-weathering processes, elemental release of seven different basalts, differing in composition, was characterized and their influence on growth and physiology of *C. metallidurans* CH34 was determined in mineral medium deficient in either calcium, iron, phosphate or magnesium. Our results clearly showed that basalt, depending on the composition, leaches different amounts of calcium, iron, magnesium and

phosphorus, which impacted growth of *C.metallidurans* CH34. Addition of basalt to iron-limiting medium restored growth as iron is released in sufficient amounts. This was not observed for phosphate and only two basalts tested, which released adequate magnesium levels, could restore growth in magnesium-limiting medium. Basalt composition analysis showed variation in the element composition of the different basalts, however, only the element release of magnesium seem to correlate with basalt composition. This could be due to the fact that during initial weathering reactions, the different primary minerals do not weather at the same rates, resulting in a non-linear ratio of release, saturation and dissolution (Nesbitt and Wilson, 1992; Gudbrandsson et al., 2011). This initial incongruent element release, results in heterogeneous partitioning of elements between the solution and formed secondary minerals, unaffected by primary mineralogy and composition (Aiuppa et al., 2000). Volcanic glass is the most susceptible component of basaltic rocks and it incongruently alters to “palagonite”, which is a mixture of these secondary precipitation reactions (Stroncik and Schmincke, 2001; Stolz and Oremland, 2011). This palagonitization consists of two stages for which the first stage is characterized by congruent dissolution of glass and basalt loses Si, Al, Fe, Mg, Ca, Na and K, while there is an passive enrichment of Ti and Fe and active enrichment of H₂O. This release of elements is also seen within our study in the abiotic samples as well as in the work of Wu et al. (2007) and Bryce et al. (2016). Although the measurements are very short term, the observed differences for magnesium could result from differences in uptake during the second stage of palagonitization, making the released magnesium unavailable for uptake and growth restoration, as the formed palagonite reacts with the released minerals and water leading to crystallization of smectite and the formation of magnesium complexes. Hereby, Si, Al, Mg and K are taken up from the solution while Ti and H₂O are released (Eggleton et al., 1987). As mentioned, the same initial release of iron was also seen by Bryce et al. (2016), where CH34 was grown in similar conditions and media as used in this study but with a different basalt composition. They, however, also saw an initial drop in total phosphate and phosphorus concentrations in the abiotic controls which was not seen in our setup. Both this phosphorus sequestration and also an increase of pH (to pH 8) was shown to halt the growth of CH34 with their basalt tested, which differed from those tested here. This increase in pH is due to glass palagonization and the release of these cations leads to metal-proton exchange, resulting in this increase (Oelkers and Gislason, 2001). This exchange can also lead to phosphate sorption onto

the rock via ligand exchange to metal oxyhydroxides as well as formation of calciumphosphates (Frossard et al., 1995; Stockmann et al., 2011). In our experiments, the pH of the solution in biotic and abiotic controls with basalt only increased from pH 7 to pH 7.6 (data not shown). This could indicate that the basalts tested in this study do not induce this pH change to the level seen with Bryce et al. (2016) or the pH change induced could be partly compensated by the medium buffering capacity in our case, leading to full growth with all basalts tested. Although there is a supplementary release of total phosphorus and phosphate from the basalt, growth limitation is thus probably not due to an increase in pH, but is maybe due to unavailability of the released phosphate (Frossard et al., 1995). This could be because of phosphate complexation of the additional phosphate released, onto the basalt surface. In addition, also other factors such as porosity, permeability, pH and surface characteristics could influence weathering of these rocks (Navarre-Sitchler and Brantley, 2007) which were not determined within this work.

Basalt can also have a positive effect on growth as shown by growth of *Pseudomonas stutzeri* VS-10, isolated from seafloor volcanic rocks, with basalt. Batch experiments indicated elevated growth of this isolate in the presence of basalt, demonstrating that most likely physical contact, or at least proximity to basaltic glass, increases growth with this type of seafloor basalt, as this leads to higher iron oxidizing activity and increased iron acquisition (Sudek et al., 2017). More subtle differences were seen as growth rates were different in the presence of different basalts tested, and this difference in basalt composition could both have a positive (SCK4) or a negative effect (SCK3) on the growth rate in complete medium. This negative effect of basalt on growth kinetics was not present in medium without calcium and no significant differences were seen here. It has already been shown that different clay types and iron mineralogy could impact bacterial activity, reduction rates as well growth kinetics (Marshall, 1975; Burdige et al., 1992; Sudek et al., 2017). In addition, significant alterations were also observed for the final density reached, for the different basalts tested. These values differed between those obtained through optical density, modelled values and flow cytometry cell counting. This could be due to the fact that optical density measurements do not take into account size or background composition changes which could impact these measurements (Nebe-von-Caron et al., 2000) and thus also modeled values and flow cytometry measurements.

Microbe–basalt interactions can accelerate elemental release and bioweathering from geologic materials, either directly through the acquisition of limiting nutrients required for biomass synthesis influencing saturation and oxidation state or indirectly by producing agents lowering pH, production of acids, complex cations through f.e. siderophores (Bennett et al., 2001; Welch et al., 2002; Gadd, 2010). In addition, as the used growth medium contains gluconate this could induce leaching in our experimental setup. This release of elements also accelerates CO₂ fixation as for every mole of calcium and magnesium released by the dissolution of basaltic silicate minerals, one mole of CO₂ is removed from the atmosphere (Wu et al., 2007) and thus consumption of these minerals by bacteria can accelerate CO₂ fixation (Barker and Banfield, 1998). Olsson-Francis et al. (2010b) showed that *C. metallidurans* CH34 did not release siderophores in 284MM-Fe supplemented with basalt, but increased transcription of ABC-type transporters putatively involved in Fe²⁺ sequestering. (Bryce et al. (2016) showed that CH34 counteracted phosphate limitation by increasing the expression of phosphate importers and utilizing intracellular phosphate reserves. However, this did not fully restore growth due to the pH increase observed with their type of basalt. Thus, *C. metallidurans* CH34 will accelerate element release directly, and not indirectly by f.e. producing siderophores, by putatively counteracting element limitation through acquisition of the nutrients, hereby increasing uptake and acquisition via upregulated transporters and influencing the saturation state of the element. In addition, it could also produce acids (Janssen et al., 2010) to increase leaching, however this was not determined within this study. In conclusion, basalt composition affects elemental release, thereby impacting element availability and uptake by bacteria, which in turn affects bacterial growth and could accelerate CO₂ fixation.

3.5 Acknowledgements

This work was supported by the European Space Agency (ESA-PRODEX), Belgian Science Policy (BELSPO) through the E-GEM/BioRock project (Bo Byloos) and the Inter-University Attraction Pole (IUAP) “μ-manager” funded by the Belgian Science Policy (BE, 305 P7/25). We thank Kai Finster and Charles Cockell for the coordination of the BIOROCK project.

Chapter 3

Chapter 4

Survival in the presence of basalt

Survival in the presence of basalt

Abstract

Microorganisms have been shown to interact with rocks and minerals to enhance the leaching of elements for sustaining their survival and allow growth. During starvation, these interactions become even more relevant for scavenging essential nutrients to support survival. Therefore, the link between nutrients and elements provided by basalt, energy status (by determining ATP levels and PHB accumulation), culturability and biofilm formation were studied for model organism *C. metallidurans*. *C. metallidurans* type strain CH34 and a PHB-negative derivative were stored during 12 weeks in mineral water supplemented with or without crushed basalt. Our results indicated that basalt had a positive effect on survival, lessening the effect of starvation, by reducing the drop in intracellular ATP levels, the accumulation of a viable but non-culturable population and PHB production. This effect was probably mediated by the release of elements such as magnesium, phosphate, potassium and sodium in the water. Cells start forming a biofilm on basalt after 4 weeks and this biofilm mode of survival differs from that seen from the planktonic one, protecting cells from the harsher environment.

Chapter partially redrafted after: Byloos, B. Van Houdt, R. Boon, N. and Leys, N. Basalt increases the culturability by utilizing PHB, impacting biofilm formation during long-term starvation of *Cupriavidus metallidurans* CH34. In preparation.

4.1 Introduction

Microorganisms have been shown to interact with rocks and minerals to enhance the leaching of elements for sustaining their survival and allowing growth. They can impact rock and mineral weathering through the production of organic acids and other ligands, which in turn impact mineral solubility, denudation and speciation (Dong, 2010). In fact, these microbe-mineral interactions are essential for soil formation through biotransformation, biochemical cycling and bioweathering (Gadd, 2010). These interactions can also lead to the formation of biofilm communities on the mineral surface, in which members will be protected from harsh environments (Harrison et al., 2005a). This transition from a planktonic to a biofilm mode, establishing more favorable biofilm structures, was shown to be evolutionary beneficial into colonizing these more nutrient-rich surfaces (Jefferson, 2004).

During starvation, these interactions become even more relevant for scavenging essential nutrients and retaining their energy status to support survival. Other strategies to support survival include transitioning into a viable but non-culturable state (VBNC), where bacteria remain active and viable but lose their culturability on media, and the accumulation of intracellular storage polymers (Pedrós-Alió et al., 1990). One of the most predominant forms of storage polymer is poly-3-hydroxyalkanoate (PHA), a highly reduced carbon and energy storage compound with its synthesis coupled to carbon and nitrogen metabolism. These storage compounds are also involved in cellular energy redox balance as these polymers act as a sink for reducing equivalents (Raberg et al., 2014; López et al., 2015). *Cupriavidus necator* and *Cupriavidus taiwanensis* are model organisms to study poly-3-hydroxybutyrate (PHB) formation and can accumulate PHB up to 90% of their cell dry weight (Chien et al., 2010). PHB accumulation can also protect *Ralstonia eutropha* from entering the VBNC state during cold storage (Northup et al., 2011). Next to cold and starvation, this VBNC state has been shown to be induced by a variety of other stresses such as pH, temperature and has been identified in 85 different species (Ayrapetyan and Oliver, 2016). Bacteria transitioning into this state become more resistant against different environmental stresses (Bogosian and Bourneuf, 2001; Oliver, 2016). Furthermore, recovery of these non-culturable bacteria has been difficult to prove, produce and reproduce (Lopez-Amoros et al., 1995; Gasol and Del Giorgio, 2000; Amor et al., 2002a; Falcioni et al., 2008; Hammes et al., 2011; Boi et al., 2015). Some of these bacteria resuscitate when

the inducing stress is removed, while the involvement of other growing bacteria and quorum sensing molecules can be important in re-culturability other (Oliver, 2016). The physiological basis for these processes is not yet fully elucidated (Kell et al., 1998; Breeuwer and Abee, 2000b; Epstein, 2013; Oliver, 2016).

The link between nutrient and element availability of rock, energy status (ATP levels and PHB accumulation), culturability and its impact on biofilm formation and microbe-mineral interactions with basalt rock have not been studied before. Therefore *C. metallidurans* cells as well as PHB-negative cells, were stored in mineral water supplemented with basaltic rock for 12 weeks and each week cell survival, physiology, biofilm formation and the elements leaching from basalt were investigated.

4.2 Material and Methods

4.2.1 Basalt composition

Basalt, an igneous volcanic rock, used in this study both for the survival and biofilm setup, was taken from the Eifel Area in Germany (SCK6 in Appendix 1). Five gram of basalt rock material, with a mesh size below 200, was prepared and used to analyze the composition of the basalt rocks, with lithium metaborate/tetraborate fusion and ICP-OES/ICP-MS analysis, and titration for FeO (Actlabs, Canada).

4.2.2 Strain and media composition

C. metallidurans strains were routinely cultured at 30°C in Tris-buffered mineral medium supplemented with 0.2% (w/v) sodium gluconate (MM284) as described previously (Mergeay et al., 1985). *E. coli* strains were routinely cultured at 37°C in Lysogeny Broth (LB). Liquid cultures were grown in the dark on a rotary shaker at 150 rpm, for culturing on agar plates 2% agar (Thermo Scientific, Oxoid) was added. When appropriate, the following chemicals (Sigma-Aldrich or Thermo Scientific) were added to the growth medium at the indicated final concentrations: kanamycin [50 µg/ml for *E. coli* (Km⁵⁰) or 1500 µg/ml for *C. metallidurans* (Km¹⁵⁰⁰)], tetracycline (20 µg/ml), 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal; 40 µg/ml), isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1 mM) and diaminopimelic acid (DAP; 1 mM). Mineral water (Chaudfontaine, Belgium) used for the preparation of the cell suspension and was 0.22 µm filter sterilised before use. Culturable bacteria were enumerated as colony forming units (CFUs) on R2A medium (2% agar; Thermo Scientific, Belgium), which is

a general medium used for environmental samples from drinking water or aqueous environments (Reasoner and Geldreich, 1985).

4.2.3 Construction of PHB-negative mutant and complementation plasmid

To investigate the effect of PHB accumulation on culturability and survival of CH34, a mutant strain was made which was unable to synthesize PHB (Figure 4.1). The *phaC₁* gene of *C. metallidurans* CH34 was amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pairs *phaC1_Fw-Rv* (Table 4.1), providing *HindIII/EcoRI* restriction sites. Afterwards, these PCR products were cloned as a *HindIII/EcoRI* fragment into the mobilizable suicide vector pK18mob. The resulting pK18mob *phaC₁* plasmid from an *E. coli* DG1 transformant selected on LB Km⁵⁰ was further confirmed by sequencing prior to amplifying of the flanking sequences of *phaC₁* by inverse PCR (Phusion High-Fidelity DNA polymerase) with primer pair *phaC1_tet_Fw-Rv* (Table 4.2), providing *BclI/BspTI* restriction sites. At the same time the *tet* gene from pACYC184 (Chang and Cohen, 1978) was amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pair *Tet_Fw-Rv* (Table 4.1), providing *BclI/BspTI* restriction sites. Afterwards, this PCR product was cloned as a *BclI/BspTI* fragment into the former inverse *phaC₁* PCR product. The resulting pK18mob-*phaC₁::tet* plasmid from an *E. coli* DG1 transformant selected on LB Tc²⁰ Km⁵⁰ was further confirmed by sequencing prior to conjugation (with *E. coli* MFDpir as donor host (Ferrieres et al., 2010)) to *C. metallidurans* CH34. The resulting transformants selected on LB Km¹⁵⁰⁰ were replica plated on LB Tc²⁰ and LB Km¹⁵⁰⁰. CH34Δ*phaC₁::tet* cells resistant to Tc²⁰ but sensitive to Km¹⁵⁰⁰ were further confirmed by sequencing. For complementation, the *phaC₁AB* locus from CH34 was amplified by PCR (Phusion High-Fidelity DNA polymerase) with primer pair *phaCAB_FW-RV* (Table 4.1), providing *XbaI/BamHI* restriction sites. Afterwards, this PCR product was cloned as a *XbaI/BamHI* fragment into pBBR1MCS2. The resulting pBBR1MCS2-*phaC₁AB* plasmid from *E. coli* DG1 transformants selected on LB Km⁵⁰ were further confirmed by sequencing prior to transformation to *C. metallidurans* CH34Δ*phaC₁::tet*. The complete locus was used to counteract any polar effect of the insertion mutation.

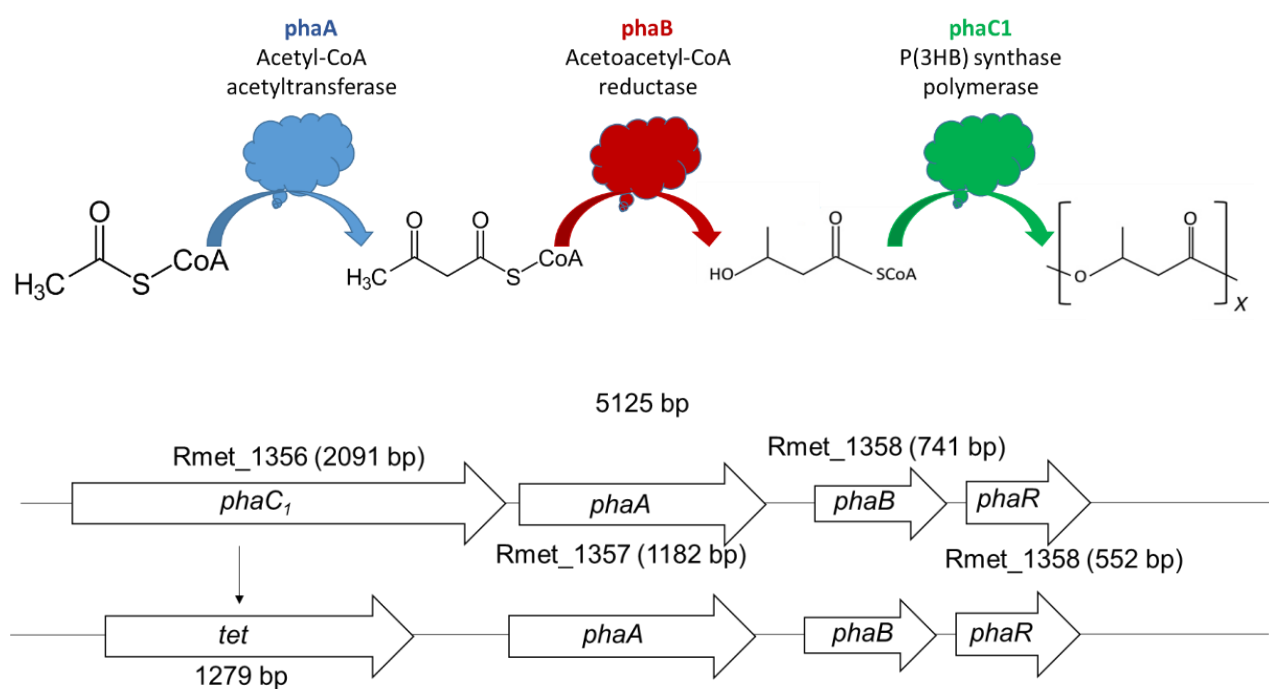


Figure 4.1: Organization of the PHB operon and associated genes in *Cupriavidus metallidurans* CH34 (bottom) and their part in the PHB assimilation pathway (top).

Table 4.1.: Primers used in this study. Restriction sites are underlined.

Primer pair	5'-3' sequence
phaC1_Fw	CTAG <u>G</u> AATT <u>C</u> GACCAGGTGCATGGCTGT
phaC1_Rv	CAGTAAGCTT <u>G</u> CCGACATCACGACCACA
phaC1_tet_Fw	GATC <u>C</u> TTAAGCATTGTTGCCTCGCAATGTA
phaC1_tet_Rv	CATCACTAGT <u>C</u> GCGCCTGAGCTTCATAAGG
Tet_FW	GATCACTAGT <u>T</u> CAGCCCCATACGATATAAG
Tet_RV	TTATC <u>T</u> TAAGTGGAGTGGTGAATCCGTTAG
phaCAB_FW	CATGT <u>C</u> TAGACGTCTGGCTGGAACGCTGAC
phaCAB_RV	TATAGGATC <u>C</u> GGGTCATGCTTGTGCACTG

4.2.4 Survival setup and analysis

Three independent cultures of *C. metallidurans* CH34 were grown to stationary phase ($OD_{600nm} \sim 1$), cells were harvested and washed three times with 10 mM $MgSO_4$ and re-suspended in mineral water (10^9 cells/ml, $OD_{600nm} = 1$). Five ml of this cell suspension was transferred to silicone cryogenic vials (VWR international, Belgium) and supplemented with or without 10 w/v% basalt, which was crushed to 1-2 mm in

size, washed in deionized water and heat sterilized beforehand. A control without cells containing only water and basalt was also prepared. The complete survival set was kept in static conditions at ambient temperature on the lab bench during 12 weeks.

Each week, a batch of the survival setup was analyzed and 1 ml of the cell suspension was transferred to sterile tubes for the following analyses: (1) two hundred microliters was used to estimate the number of viable cells plating a serial dilution on R2A agar, (2) two hundred microliters was used for flow cytometry analysis, ATP and PHB measurements and (3) six hundred microliters was used to measure pH. The remaining 4 ml of the solution was used for ICP-OES analysis (see section ICP-OES).

4.2.4.1 Sonication

After analysis of the planktonic fraction (described in the previous paragraph) also the biofilm fraction present on the crushed basalt was analysed. Therefore, 5 ml filtered mineral water was added to the silicone vials, after removal of the rest of the supernatant, and the solution was probe sonicated for 3 min at 20kHz, 4W at low-intensity. Thereby cells in the biofilm are released in the water and could be analyzed as described in the previous paragraph. The protocol for sonication was determined before the survival experiment and was based on previously described protocols (Kobayashi et al., 2009) and was found to be optimal at the conditions described above.

4.2.4.2 Flow cytometry

Samples were stained with flow cytometry according to the optimized procedures described in Buysschaert et al. (2016), Van Nevel et al. (2013) and SLMB (2012) (see also section 3.2.3). Cell suspensions were diluted 10,000 times in 0.2 μ m filtered Evian mineral water as this gave the lowest background fluorescence. Next, SGPI dye (SYBR Green (SG) (Sigma Aldrich; final concentration of 1x) and PI (Sigma Aldrich; final concentration of 1 μ M) was added and cell suspensions were incubated at 35°C for 13 min. Work solution of SGPI was prepared in DMSO and kept at 4°C. These were prepared from stock solutions in DMSO kept at -20°C. Sybr Green (SG) was used to stain all cells as this stain can enter both intact and damaged cells independent from their physiological state. Propidium iodide (PI) was used as a cell-impermeable nucleic acid stain that is not able to cross the membrane due to its size and charge and can therefore be used as an indicator of membrane permeability (Berney et al., 2007).

Stained bacterial suspensions were analyzed on an Accuri C6 (BD, Erembodegem) with a blue (488 nm, 20mW) and red (640 nm, 14.7 mW) laser which was calibrated according to the manufacturer's recommendation. Standard optical filters were used and included FL-1 (530/30 nm) and FL-3 (670 LP) for the blue laser. The dye SYBR Green was detected with FL-1, PI was detected on FL-3. A quality control with 6 and 8 peaks fluorescent beads (by manufacturer BD, Erembodegem) and cleaning cycle was performed prior to experiments to assess both the accuracy (bead count and position) and the cleanliness of the machine. Samples were analyzed using the Accuri C6 software (version 1.0.264.21). The flow files were uploaded to the FlowCyt database.

4.2.4.3 Intracellular ATP

To measure intracellular ATP levels the BIOTHEMA (Handen, Sweden) intracellular ATP kit HS was used and adapted from the manufacturer's protocol for smaller volumes. Cell suspensions were diluted 1/100 and 25 µl of this dilution was added in a cuvette. Twenty five microliters of ATP eliminating agent was added and the cuvette was left to incubate for 10 min to degrade all the extracellular ATP. Twenty five microliters of cell lysis 'extractant BS' was added to the cell solution, vortexed and immediately thereafter 200 µl 'ATP reagent HS' was added. Luminescence was measured immediately after (Kikkoman Lumitester c-100). Five microliters of a 100 nmol/l ATP standard was added as an internal control and light emission (luminescence) was measured again. The average intracellular ATP concentration per cell was calculated as follows: $(I_{\text{sample}} / (I_{\text{standard+sample}} - I_{\text{sample}})) / \text{TC}$, where I is the luminescent emission measured and TC the total cell count as measured by flow cytometry after SG staining.

4.2.4.4 Intracellular PHB

To measure intracellular PHB levels, a Nile Red staining protocol was developed, based on Degelau et al. (1995), for *Cupriavidus metallidurans* CH34 and adapted to our test conditions. Nile red binds selectively to non-polar lipid droplets inside cells and can be used to detect the presence of storage lipids (PHA/PHB) via fluorescence spectrophotometry (Greenspan and Fowler, 1985; Johnson and Spence, 2010). A Nile red working solution (200 mg/l) was prepared from a 100 mg/100 ml Nile red stock solution in DMSO and stored at -20°C. Ten µl of the cell solution was diluted in 100 µl sterile Evian mineral water in a microtiter plate, five µl of the work solution was added and incubated for 30 min at 30°C. Water without cells but with dye was used as a

negative control and water without cells and without dye was used for background correction (blanco). Afterwards fluorescence of the lipid-bound Nile red was determined with a Thermo Scientific™ Fluoroskan Ascent™ Microplate Fluorometer. Nile red solution has an excitation peak at 544 nm and an emission peak at 590 nm. The normalized amount of intracellular PHB per cell was then calculated with the following formula:

$$\frac{(FI_{590\text{ nm},10\text{ }\mu\text{l sample}} - FI_{590\text{ nm},\text{negative control}} - FI_{590\text{ nm},\text{blanco}})/OD_{600\text{ nm}}}{\text{total cell count (with SG) in measured sample}}$$

4.2.5 Biofilm quantification and visualization setup and analysis

Three independent cultures of *C. metallidurans* CH34 were grown to stationary phase ($OD_{600\text{ nm}} \sim 1$), cells were harvested and washed three times with 10 mM $MgSO_4$ and re-suspended in mineral water (10^9 cells/ml, $OD_{600\text{ nm}} = 1$). Five ml of this cell suspension was transferred to a 6-well microtiter plate (Biogreiner, Germany) and supplemented with a square basalt slide (1 cm x 1 cm x 1 mm; which was cut in these dimensions from the same basalt as the crushed basalt), which were washed in deionized water and heat sterilized beforehand. Next, the microtiter plate was covered with Parafilm M® (Bemis, VWR) and kept in static conditions at ambient temperature on the lab bench during 12 weeks.

To quantify biofilm formation, three different basalt slides were analyzed, each split into two equal parts, and two different staining protocols were used adjusted from Peeters et al. (2008). For cFDA staining, 5 ml of filtered Evian and 50 μl of cFDA work solution (final concentration 10 μM) were added and incubated in the dark (37°C). Fluorescence was measured after 20 min (λ_{ex} : 488 nm and λ_{em} : 515 nm). The cFDA (Sigma) work solution was prepared in DMSO (1mM) and kept at 4°C. For crystal violet (CV) staining, 5 ml of a 0.03% CV solution was added (after wash step detailed above). After 10 min the excess CV was removed by adding 5 ml of milliQ water and rinsing. Finally, bound CV was released by adding 5 ml of 100% ethanol, incubation of 10 min and absorbance measurement at 590 nm.

For biofilm visualization, one basalt slide was divided into 4 parts for each of the stains tested and put in a 24 well MTP. For SG and cFDA staining 10 μl of the work solution was added to 1 ml of Evian filtered water. Calcofluor, which specifically stains (1→4)- and (1→3)-beta-D-glucan polysaccharides, stock solution was diluted 1/10 in 1 ml

filtered Evian solution of which the stock solution was kept at room temperature (Neu et al., 2002). 1 ml of SYPRO Ruby stock solution, staining most classes of proteins, was added to the well (Tremblay et al., 2013). Afterwards the plate was left to incubate for 30 min at RT protected from light and afterwards visualized with an Eclipse Ti microscope (NIKON) equipped with a 40 × Plan Fluor objective (NA 0.6) and an Andor Ixon EMCCD camera for which the basalt slide was attached to a glass slide ((SG; λ_{ex} : 497 nm and λ_{em} : 520 nm), (cFDA λ_{ex} : 492 nm and λ_{em} : 517 nm), (calcofluor; λ_{ex} : 365 nm and λ_{em} : 435 nm) and (SYPRO Ruby λ_{ex} : 470 nm and λ_{em} : 618 nm)).

4.2.6 ICP-OES

At the end of the experiment, the in-organic element concentrations in the water were measured to compare changes in water chemistry induced by the rocks and cells during the experiment. Four milliliters of the supernatant from the vials with and without basalt fragments was taken from the tubes and centrifuged (10 000 x g; 15 min, 20°C) to pellet the cells. This supernatant was then filtered through a 0.22 μm filter to remove particles and 20 μl of 70% nitric acid was added before final inorganic element concentrations in the cell-free supernatant were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Actlabs, Canada).

4.2.7 Statistical analysis

For the statistical analysis of the data the GraphPad Prism (version 7.0) software package was used. For all the data of the cell suspension, normal distribution was assumed and two way ANOVA was used with Dunnet post-hoc testing to compare data from the start ($\alpha=0.05$) and Tukey post-hoc testing to compare the different categories ($\alpha=0.05$). For the biofilm quantification assay two-tailed one way ANOVA was used with Tukey post-hoc testing ($\alpha=0.05$). For the mutant PHB negative cells comparison, two-tailed one way ANOVA was used with Tukey post-hoc testing ($\alpha=0.05$).

4.3 Results

4.3.1 The effect of basalt on culturability, membrane permeability and energy status of *C. metallidurans* during 12-week storage

A cell suspension of 10^9 *C. metallidurans* cells per ml was stored in mineral water with or without crushed basalt during a period of 12 weeks. On a weekly basis a sample was taken and cell viability, culturability and energy status (ATP and PHB) were determined (Figure 4.2 and 4.3). During the 12-week storage, culturability gradually

decreased 2 log CFU/ml ($p < 0.001$) compared to the initial cell number. In the presence of basalt, viability decreased less, with a 0.5 log reduction after 12 weeks ($p = 0.0048$), and this positive effect of basalt on culturability ($p < 0.0001$) became apparent from week 7 (Figure 4.2). The impact of starvation conditions and the presence of basalt was also analyzed with flow cytometry by staining with specific functional dyes (Figure 4.2). The total cell numbers (measured with SG) as well as the number of intact cells (SG positive cells when stained with both SG and PI) remained constant throughout the 12-week experiment, irrespective of the presence of basalt, and were comparable with the initial numbers (Figure 4.2). The fraction of cells with increased membrane permeability (PI positive cells when stained with both SG and PI, Figure 4.2) increased gradually during the experiment and was inversely proportional to the culturability. The presence of basalt exerted a positive effect and decreased this fraction by 10% after 12 weeks ($p = 0.0002$). Again, this positive effect became apparent from week 7 ($p = 0.02$).

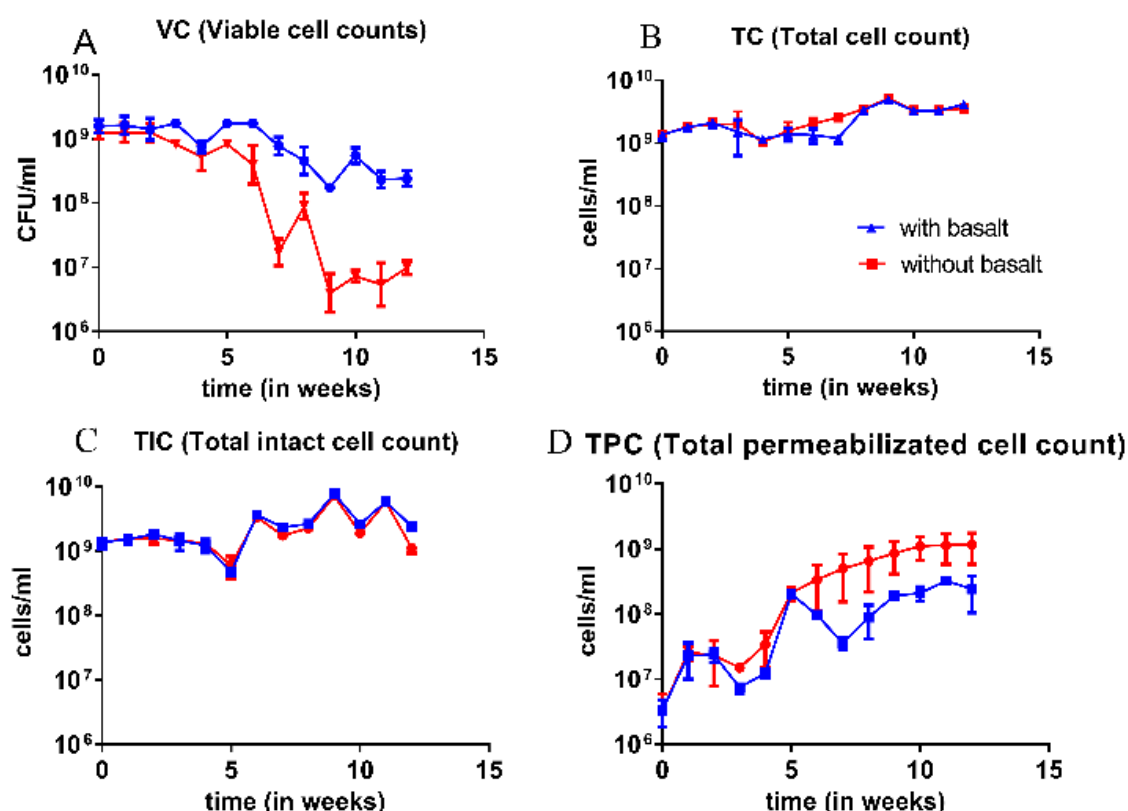


Figure 4.2: Culturability (A), total (B), intact (C) and permeable (D) cell count of planktonic *C. metallidurans* CH34 during 12-week storage in mineral water with (blue) and without (red) basalt. Each time point is represented as a dot of the mean with SD.

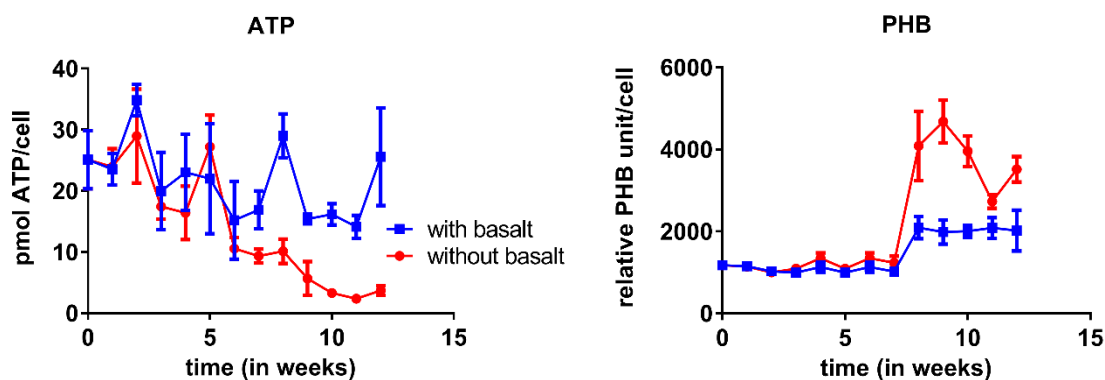


Figure 4.3: ATP and PHB content of planktonic *C. metallidurans* CH34 during 12-week storage in mineral water with (blue) and without (red) basalt. Each time point is represented as a dot of the mean with SD.

Intracellular ATP as well as PHB levels were measured to determine the energy status of the cells (Figure 4.3). In water, ATP levels declined progressively over the 12-week experiment, leading to a 25-fold decrease ($p < 0.0001$). Basalt counteracted this decline and ATP levels remained comparable to the start conditions, resulting in a significant difference between the ATP level of cells in the absence and presence of basalt after 12 weeks ($p = 0.0009$). The PHB content per cell increased after 8 weeks of storage and cells in water contained 4 times more PHB ($p < 0.0001$) compared to the start (Figure 4.3). Basalt reduced this accumulation and cells only doubled the amount of PHB compared to the start of the experiment, leading to a significant difference in PHB content of cells with and without basalt after 12 weeks ($p = 0.01$).

4.3.2 The effect of PHB production on culturability, membrane permeability and energy status

To investigate the effect of PHB production, a *phaC1* mutant deficient in PHB accumulation was constructed and analyzed. After 12 weeks of storage in water culturability of the PHB-negative strain decreased 2 log, which was comparable with the decrease observed for the parental and complementation strain (Figure 4.4). In the presence of basalt, culturability decreased 0.5 log less, resulting in a 0.5 log difference between the absence and presence of basalt ($p = 0.01$). However, this decrease was less pronounced than for the parental and complementation strain (2 log difference between absence and presence of basalt for both). Enumeration of the total cell population by flow cytometry indicated that the PHB-negative total cell population remained constant during the 12-week storage period both in the absence and presence of basalt, which is similar to the parental and complementation strain (Figure

4.4). More intact cells were observed in the presence of basalt for the parental and complementation strain. This apparent positive effect of basalt was not observed for the PHB-negative strain. The fraction of permeable cells decreased for the parental and complementation strain in the presence of basalt and this effect was smaller, but not significantly different for the PHB-negative strain.

The intracellular ATP level in PHB-negative cells was comparable in the presence and absence of basalt and corresponded to that observed for parental cells in the absence of basalt (Figure 4.4).

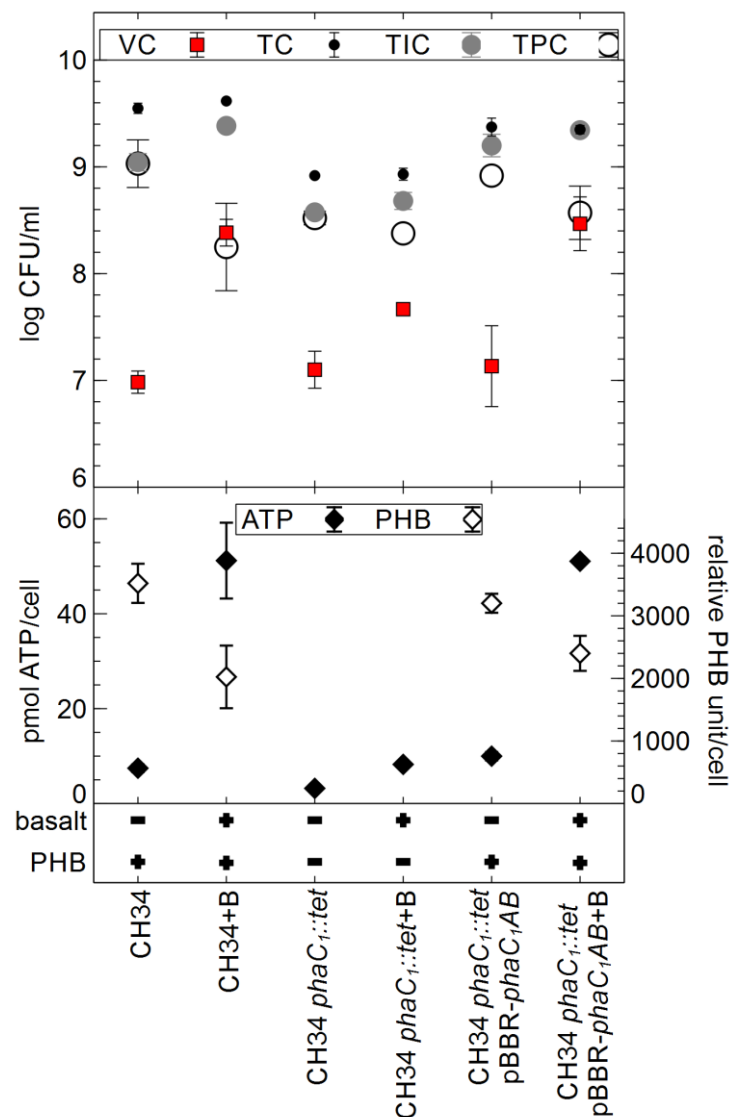


Figure 4.4: Culturable fraction (square in red, upper part) of PHB negative cells measured on R2A medium. Total fraction (black circle, upper part), intact (grey circle, upper part) and non-intact fraction (white circle, upper part) of planktonic PHB negative cells after the 12 week survival experiment measured with flow cytometry. Energy status of planktonic cell fractions measured by ATP (black diamond, lower part) and PHB (white diamond, lower part) content during the 12 week experiment.

4.3.3 Biofilm formation on basalt

4.3.3.1 Culturability, membrane permeability and energy status of biofilm cells

On a weekly basis a sample was taken and cell viability, culturability and energy status (ATP and PHB) of the biofilm cells on basalt were determined (Figure 4.5 and 4.6). Culturability declined over the 12-week experiment, leading to a 1 log reduction after 12 weeks compared to the initial start concentration ($p=0.012$). The culturable fraction is 1 log less compared to that of the planktonic fraction ($p<0.0001$). The total biofilm cell number, measured with flow cytometry, increased significantly at 7 weeks, compared to the start ($p<0.005$) but not compared to that seen after 12 weeks (Figure 4.5). The amount of total cells measured is comparable to that seen for the CH34 planktonic fraction. The fraction intact biofilm cells remained constant whereas the fraction of biofilm cells with increased membrane permeability declined ($p<0.0001$) compared to the initial numbers. The amount of intact and non-intact cells is again comparable to that seen for the CH34 planktonic fraction.

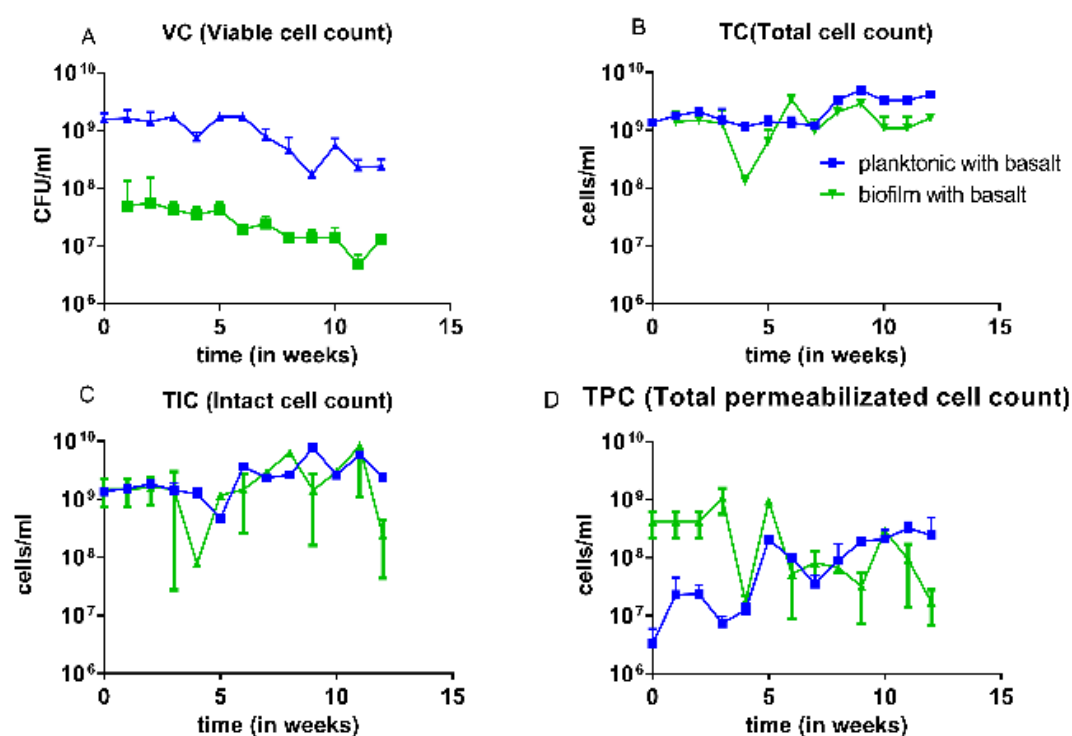


Figure 4.5: Culturability (A), total (B), intact (C) and permeabilized (D) cell count of biofilm (green) and planktonic (blue) *C. metallidurans* CH34 cells during 12-week storage. Each time point is represented as a dot of the mean with SD.

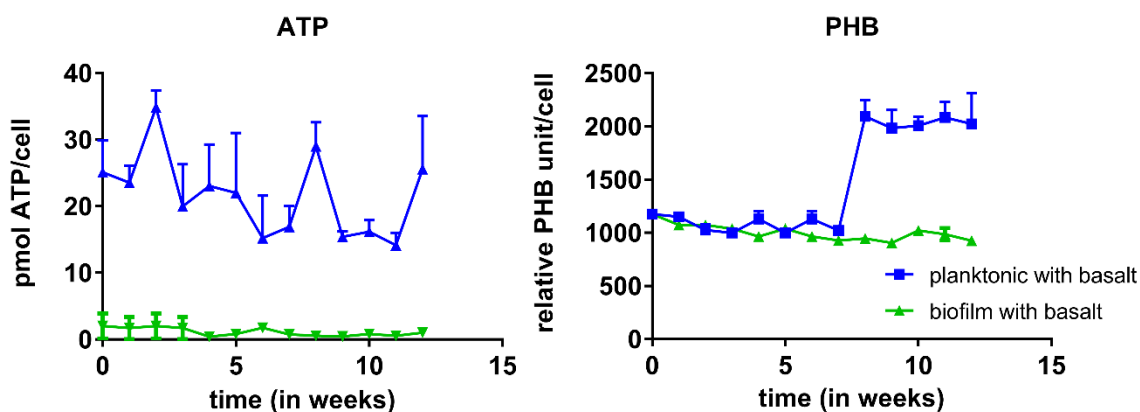


Figure 4.6: ATP and PHB of biofilm (green) and planktonic (blue) *C. metallidurans* CH34 cells during 12-week storage content. Each time point is represented as a dot of the mean with SD.

The ATP level of the biofilm cells remained stable throughout the 12-week experiment, however, levels were 25 times lower than measured for the planktonic fraction. For PHB, biofilm cells contained slightly less compared to the start ($p=0.04$) and two times lower than measured for the CH34 planktonic fraction.

4.3.3.2 Biofilm visualization and quantification

To allow better visualization and quantification of the biofilm formation, a cell suspension of 10^9 *C. metallidurans* cells per ml was stored in mineral water with or without a basalt slide in microtiter plates during a period of 12 weeks. On a weekly basis slides were sacrificed and biofilm formation was quantified via cFDA and crystal violet (CV) assays (Figure 4.7). Significant biofilm formation was quantified from week 3 with the cFDA ($p=0.00015$) and week 4 with CV protocol ($p<0.0001$). The amount of fluorescence/optic density remained constant, on subsequent biofilm batches, both with cFDA and CV quantification respectively, significantly differencing ($p<0.0001$ for both) from the start.

Biofilm development and attachment were also investigated with fluorescence microscopy and staining with SG, cFDA, SUPRO Ruby and Calcofluor (Figure 4.8). SG staining showed already after the first week cells on the surface of the basalt. After 3 weeks no individual cells could be seen and extracellular staining was observed, which could indicate the presence of eDNA. Calcofluor (Calco), which specifically stains polysaccharides, showed the presence of fluorescent complexes after 3 weeks.

For cFDA, converted by esterases to its fluorescent product cF, and the SYPRO Ruby protein stain fluorescent complexes could be observed after 5 weeks of storage.

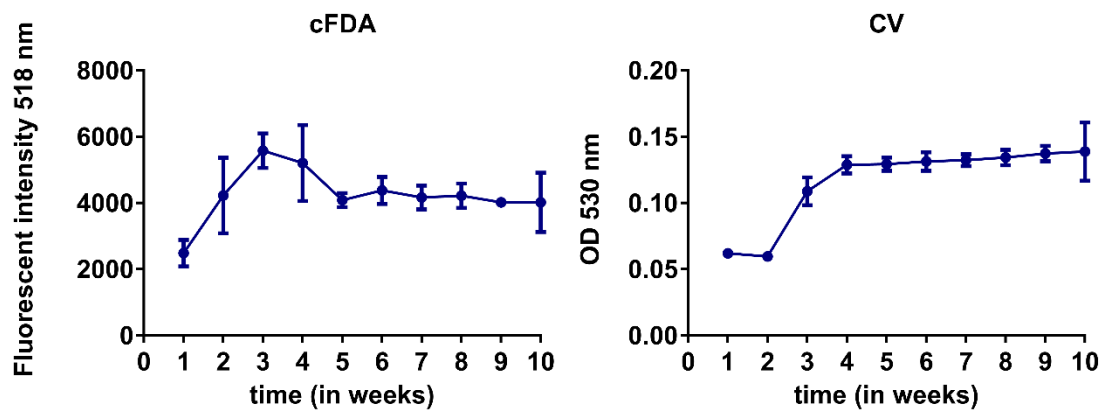


Figure 4.7: Quantification of biofilm formation on basalt with cFDA fluorescence and crystal violet (CV) assays. Each time point is represented as a dot of the mean with SD.

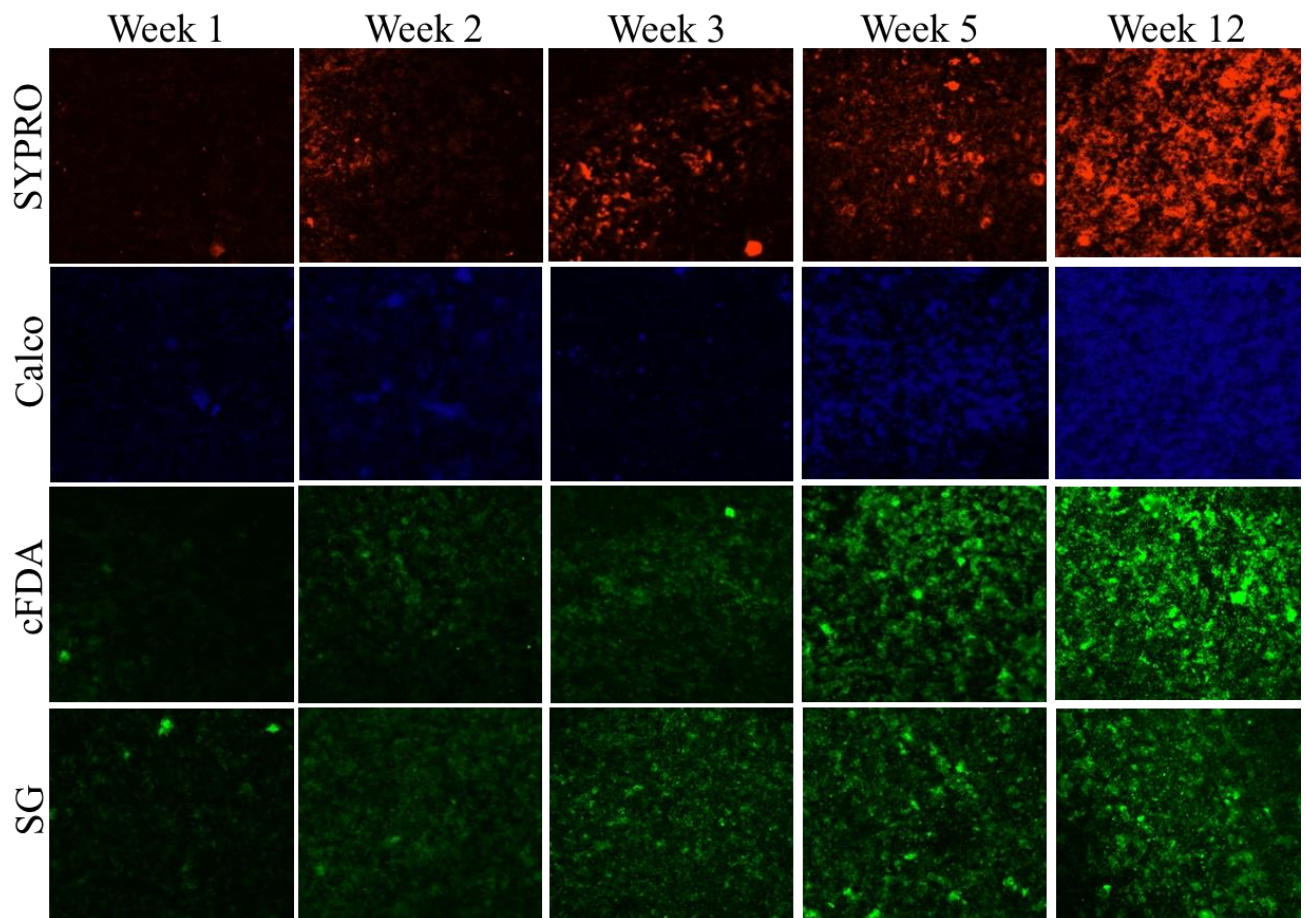


Figure 4.8: Fluorescence microscopy of biofilm formation on basalt at 200x magnification after 1, 2, 3, 5 and 12 weeks of storage using SYPRO Ruby (SYPRO, protein stain), Calcofluor (Calco, polysaccharide stain), cFDA (cFDA, esterase stain) and Sybr Green (SG, DNA stain).

4.3.3.3 Effect of PHB production on culturability, membrane permeability and energy status of biofilm cells

To investigate the effect of PHB production on biofilm formation, a *phaC1* mutant deficient in PHB accumulation was constructed and analyzed. After 12 weeks of storage in water, culturability of the PHB-negative biofilm cells decreased 1 log compared to the start concentration, corresponding to the drop seen for the parental and complemented biofilm cells (Figure 4.9). This led to an 0.5 log lower culturable fraction of PHB-negative cells compared to the parental and complemented biofilm cells ($p=0.008$). The total cell number of the PHB-negative biofilm cells was 1 log lower than measured for the parental and complemented biofilm cells ($p=0.0002$). Furthermore, the fraction of intact biofilm cells decreased ($p=0.003$) while more cells were permeabilized ($p=0.0004$) compared to the parental and complemented biofilm cells.

After 12 weeks, ATP levels dropped in the PHB negative cells, on average 3 times compared to planktonic PHB negative cells, to levels comparable to those seen in the parental and biofilm cells (Figure 4.9).

4.3.4 The effect of basalt on element release

ICP-OES was performed to quantify element release in the solution and evaluate the possible impact of *C. metallidurans* on the leaching of elements from basalt (Figure 4.10). Abiotic storage of basalt in mineral water increased the concentration of potassium, sodium, phosphate and phosphorus, and decreased the concentration of calcium. Storage of *C. metallidurans* in the presence of basalt indicated that cells consumed potassium, sodium, phosphate and phosphorus, and released magnesium and calcium in the water.

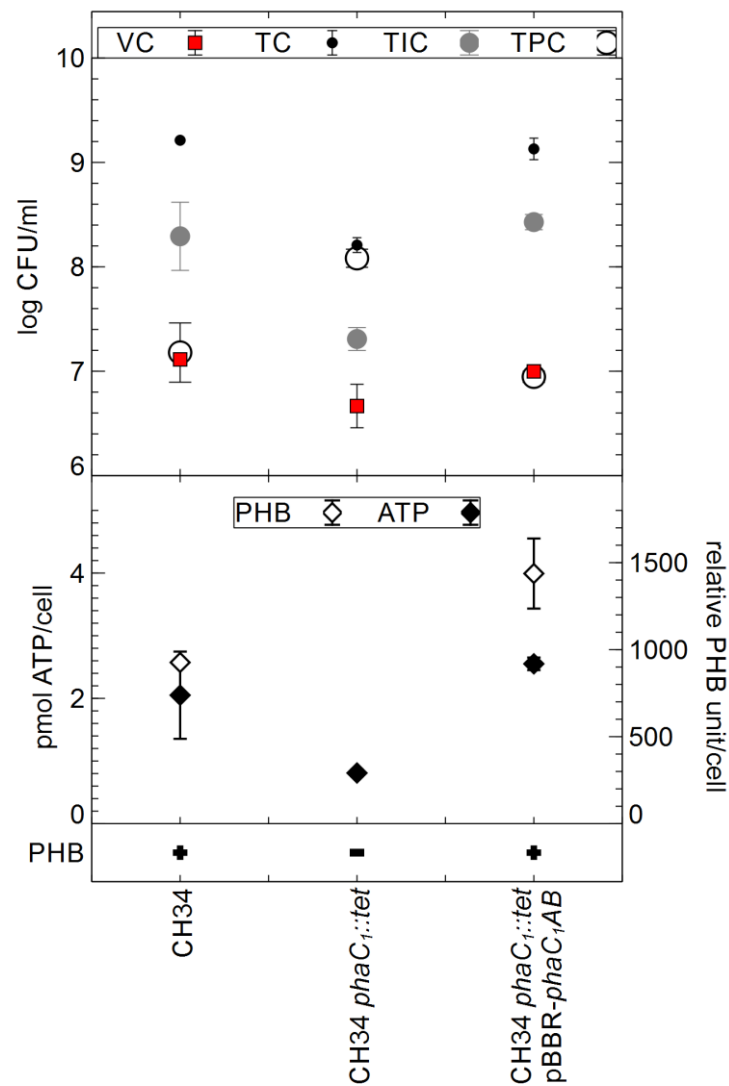


Figure 4.9: Culturable fraction (square in red, upper part) of biofilm PHB negative cells after 12 weeks, determined on R2A medium. The total fraction (black circle, upper part), intact (grey circle, upper part) and non-intact fraction (white circle, upper part) of planktonic PHB-negative cells after the 12 week survival experiment measured with flow cytometry. Energy status of planktonic cell fractions measured by ATP (black diamond, lower part) and PHB (white diamond, lower part) content during the 12 week experiment.

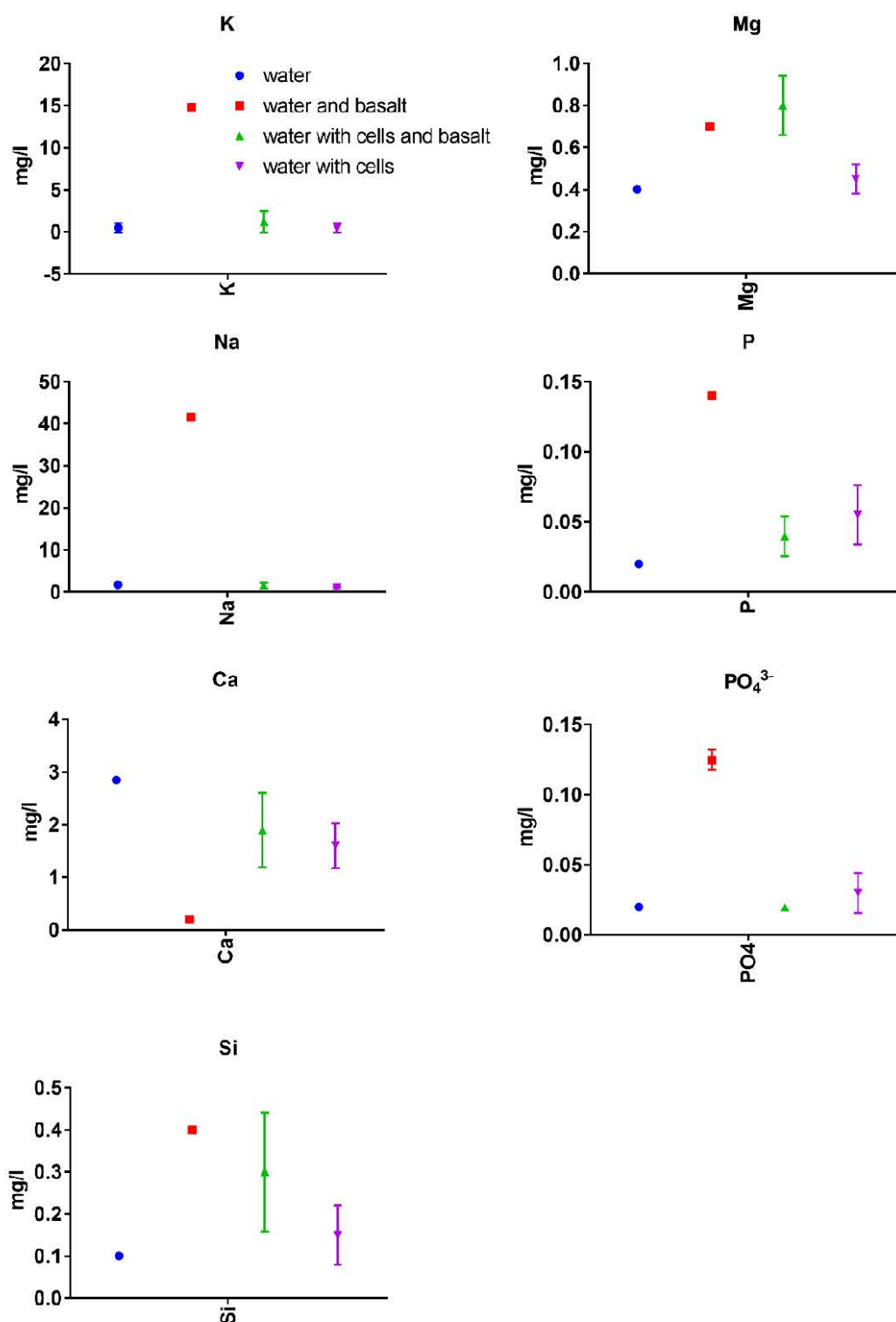


Figure 4.10: Results of the ICP-OES analysis for the potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), calcium (Ca), phosphate (PO_4^{3-}), silicium (Si) expressed as the concentration in the suspension (in mg/l).

4.4 Discussion

To evaluate the potential impact of basalt on bacterial starvation, energy status and viability *C. metallidurans* CH34 was stored in mineral water supplemented with basaltic rock for 12 weeks and each week culturability, total, intact and permeabilized cell fractions as well as energy status, biofilm formation and elemental release from basalt were investigated.

As expected, long-term storage had a significant impact on physiology, energy status and culturability of CH34 cells. Less cells were culturable, cells contained less ATP and accumulated PHB after 12 weeks compared to the start of the experiment and in addition contained more permeabilized cells. The CH34 cells thus reduce their 'immediate operational' energy levels (ATP) but increase their energy storage levels (PHB) when put in these survival conditions. The drop in culturability seen in water without basalt, after 7 weeks, thus coincides with a 25-fold decrease in energy levels (ATP), and a four-fold increase in energy stock levels (PHB) over the 12 week experiment. It was already been shown that *C. metallidurans* and closely related *Ralstonia pickettii* isolates could survive in mineral water with high silver concentrations for almost two years, showing a decline in culturability (Mijnendonckx et al., 2013). Decreased culturability, without a decline in cell numbers, indicated that the cells in these oligotrophic conditions transition into a less culturable but still viable state (VBNC). This has already been shown in other experiments in similar oligotrophic conditions (Kell et al., 1998; Oliver, 2005). *Ralstonia solanacearum* also showed this transition into the VBNC state while present in river water (Álvarez et al., 2008). *R. solanacearum* also transitions into this VBNC state when exposed to high concentrations of copper, low temperature or in soil (Grey and Steck, 2001; van Overbeek et al., 2004; Caruso et al., 2005). The presence of basalt had a positive effect on culturability, leading to only a 0.5 log reduction of culturability on R2A medium. This effect of basalt, lessening the effect of starvation, was also seen for the energy status, as these cells in the presence of basalt also contain more ATP and less are permeabilized while accumulating less PHB compared to cells in water.

To investigate the relation between PHB, survival and basalt further, a CH34 mutant, unable to synthesize PHB was constructed. Culturability decreased in the PHB-negative cells corresponding to the response seen with the parental strain. The

presence of basalt could however not fully counteract this decrease as observed for the parental strain. Previous studies showed that cells accumulate PHB in nutrient-poor environments such as mineral water (Kadouri et al., 2005). PHB accumulation could result from metabolic redirecting of proteome or lipid cellular fractions as seen in the closely related *Ralstonia eutropha* strain H16 (Brigham et al., 2010; Sharma et al., 2016). Other factors such as exposure to stress conditions (Povolo and Casella, 2000; Rojas et al., 2011) and phosphate limitation (Shang et al., 2003; Budde et al., 2011) can trigger PHB accumulation as well. Accumulation of PHB can, as an energy reserve, have a positive effect during starvation (Wang and Bakken, 1998), and the ability for PHB metabolism by producing and degrading PHB improves fitness of rhizospheric bacteria as it stabilizes the intracellular redox conditions and is able to compensate TCA cycle inhibition in low oxygen conditions (Anderson and Dawes, 1990; Dunn, 1998). This PHB utilization leads to a more balanced growth rate that can give a competitive advantage in surviving these environments over organisms without this storage capability (Beun et al., 2002). Impaired PHB accumulation in *Ralstonia eutropha*, by construction of PHB negative cells, leads to loss of culturability of these cells compared to the wild type (López et al., 1995; Nowroth et al., 2016). This effect on culturability, however, is not seen for our PHB negative cells and they are as culturable as the parental strain in water (Chapter 5). Basalt could however partly restore culturability of these cells, but not to the level observed in the parental strain, showing again that basalt could reduce the effects of starvation through element release. The exact role of PHB accumulation however remains to be elucidated.

Next to the planktonic fraction, cells also start forming a biofilm on basalt. Cells were already present on basalt after 1 week, while polysaccharides and proteins complexes were observed after 3 weeks. This was in correspondence with cFDA and CV quantification which significantly increases after 3 and 4 weeks, respectively and onwards, indicating that biofilm started around week 3. Results between the cFDA and CV quantification as well as fluorescent visualization differed, however showing that differences exist between the two protocols. This difference can be due to the fact that cFDA, as enzymatic dye, allows more specific biofilm staining while CV stains all negatively charged molecules and thus less specific (Peeters et al., 2008). Both fluorescent quantification and qualification results are difficult to correlate and is depended on the organisms and substrate studied (Decker, 2001; Hannig et al., 2010).

When the physiology of these cells is determined, the biofilm mode of growth impacts both culturability as well ATP and PHB levels, showing that the biofilm mode differs from that seen for the planktonic fraction. The culturable biofilm fraction was 1 log lower compared to the culturable planktonic fraction, while the total cell amount remains at the same level for both the biofilm and planktonic fraction. Other studies also showed this difference between culturable and total cell amount in biofilm cells as well as the difference between biofilm and planktonic fractions (Prakash et al., 2003; Manuel et al., 2007). However, less cells are permeabilized, and ATP levels do not decline, but remain at a lower level than that seen for the planktonic fraction, which may indicate that once these cell transition into the biofilm mode of growth, these cells are protected from the harsher survival conditions in the planktonic fraction (Marshall, 1988; Prakash et al., 2003). Intracellular PHB content is, however, slightly lower than measured for the planktonic cell fraction. The total cell amount of the PHB negative biofilm cells declined one log compared to the total biofilm CH34 fraction showing that less PHB negative cells can transition into the biofilm mode of growth. These findings may thus indicate that PHB could play a role in biofilm formation. It has already been shown that *Sinorhizobium meliloti* PHB negative cells can no longer produce the extracellular polysaccharide (EPS) succinoglycan (Aneja et al., 2004). These lower cell amounts are also reflected by lower culturability and lower ATP content of PHB negative biofilm cells. This might indicate that PHB accumulation could be involved in biofilm formation, also shown by Tribelli and López (2011).

For the elemental analysis, basalt released potassium, sodium, phosphate and phosphorus into the water while calcium was removed from the water, probably due to complexation on basalt (Stockmann et al., 2011). These element release thus impacted the survival of cells, as we could show that cells in the presence of basalt take up potassium, sodium, phosphate and phosphorus, while more magnesium is found in water after the 12 week experiment as well as calcium is released in the water.

Our results indicated that basalt had a positive effect on survival, probably by releasing elements such as potassium, sodium, phosphate and phosphorus that, counteract some of the detrimental effects of starvation. In the presence of basalt, a larger fraction of the starved population remained viable, contained a larger culturable fraction and a smaller fraction of permeabilized cells. Moreover, these cells contained more ATP and accumulated less PHB. Basalt thus reduces the transition into a VBNC state. Cells also

start forming a biofilm on basalt, with a larger fractions of cells that remains intact compared to the planktonic mode as well as contain less ATP, which will protect the cells from the harsher planktonic environment. Finally, PHB could potentially be involved within this biofilm formation.

4.5 Acknowledgements

This work was supported by the European Space Agency (ESA-PRODEX), Belgian Science Policy (BELSPO) through the E-GEM/BioRock project (Bo Byloos) and the Inter-University Attraction Pole (IUAP) “ μ -manager” funded by the Belgian Science Policy (BE, 305 P7/25).

Chapter 5

Impact of space conditions on survival in the presence of basalt

Impact of space conditions on survival in the presence of basalt

Abstract

Microbe-mineral interactions have become of interest for space exploration as microorganisms could be used to biomine from extra-terrestrial material and extract elements useful as micronutrients in life support systems. This research aimed to identify the impact of space flight on the long-term survival of *Cupriavidus metallidurans* CH34 in mineral water and the interaction with basalt, a lunar-type rock in preparation for the ESA spaceflight experiment, BIOROCK. Therefore, *C. metallidurans* cells were suspended in mineral water supplemented with or without crushed basalt and sent for three months on board the Russian FOTON-M4 capsule. Long-term storage had a significant impact on cell physiology and energy status as 60% of cells stored on ground lost their cell membrane potential, only 17% were still active, average ATP levels per cell were significantly lower and culturability dropped to 1%. The cells stored in the presence of basalt and exposed to space flight conditions during storage, however, showed less dramatic changes in physiology, with only 16% of the cells lost their cell membrane potential and 24% were still active, leading to a higher culturability (50%) and indicating a general positive effect of basalt and space flight on survival. Microbe-mineral interactions and biofilm formation were also altered by spaceflight as less biofilm was formed on the basalt during flight conditions. Leaching from basalt also changed, showing that flight conditions thus could counteract some of the detrimental effects observed after the three month storage conditions.

Chapter partially redrafted after: Byloos, B. Coninx, I. Van Hoey, O. Cockell, C. Nicholsson, N. Ilyin, V. Van Houdt, R. Boon, N. and Leys, N. (2017). The impact of survival conditions on the interaction and culturability of *Cupriavidus metallidurans* CH34 with basalt, a volcanic moon analog rock. *Frontiers in Microbiology*. 8:671.

5.1 Introduction

Microorganisms can interact with rocks and minerals to enhance leaching of elements for sustaining their survival and growth. They can impact rock and mineral weathering through production of organic acids and other ligands, which in turn impact mineral solubility, denudation and speciation (Dong, 2010). These microbe-mineral interactions are in fact essential for soil formation through biotransformation, biochemical cycling and bioweathering (Gadd, 2010). In addition, they can be useful for and have already been applied in industry. For example, acidophilic iron- and sulfur oxidizing bacteria are used in bio-mining applications to oxidize copper and gold sulfidic bonds in order to solubilize and recover the economically interesting metals from the ores (Uboldini et al., 2000a). These interactions can also lead to the formation of biofilm communities on the mineral surface, in which members will be protected from harsh environments (Harrison et al., 2005a).

Microbe-mineral interactions have also become of interest for space exploration missions. At the moment, human presence in space needs to be fully supported from Earth. To reduce the costs and the dependency for supplies from Earth for future more distant space missions, current research is investigating if supplies can be generated from endogenous material on planets and asteroids, such as the regolith and rocks. Microorganisms can be used in this process of in situ resource utilization (ISRU) to extract useful elements that could be applied as fertilizers in a life support system and in the formation of fertile soil for plant cultivation (Cockell, 2010).

Since space conditions have been shown to cause many changes in bacterial physiology, including changes in motility and biofilm formation (Brown et al., 2002; Leys et al., 2004; Horneck et al., 2010; Leroy et al., 2010; Kim et al., 2013c), these conditions may also influence microbe-mineral interactions as microgravity eliminates mass-driven convection and only diffusion can impact element release and availability as well as alter microbe-mineral contact (János et al., 2002). To evaluate the possibility of microbe-based ISRU, the potential impact of space environmental conditions such as microgravity and radiation on microbe-mineral interactions need to be studied.

Our study aimed at investigating the influence of space conditions on these microbe-mineral interactions, by testing the impact of space flight conditions on the survival and biofilm formation of the bacterium *Cupriavidus metallidurans* CH34 in mineral water

supplemented with basalt. It was already shown that *C. metallidurans* and closely related *Ralstonia pickettii* isolates could survive in mineral water for at least two years, even in the presence of antimicrobial silver (Mijnendonckx et al., 2013).

In order to prepare for potential future feasibility studies of biomining in space (the ESA BIOROCK experiment), preliminary tests were performed to assess the impact of flight conditions on an inactive bacterial inoculum, and its interactions with basalt rock. Therefore *C. metallidurans* CH34 was stored in mineral water supplemented with basaltic rock and sent on board of the FOTON-M4 capsule for three months. After flight, cell survival, physiology, biofilm formation and the elements leaching from basalt were investigated

5.2 Material and Methods

5.2.1 Strain and media composition

Cupriavidus metallidurans type strain CH34 (Mergeay et al., 1985) was cultured at 30°C on a shaker in dark, aerobic conditions in a Tris buffered mineral (284MM) medium containing 2 g/l sodium gluconate (Merck) as sole and more selective carbon source (Mergeay et al., 1985). Mineral water (Chaudfontaine, Belgium) was used to prepare the cell's suspensions. Culturable bacteria were enumerated as CFUs on R2A medium (2% agar; Thermo Scientific, Belgium) and 284MM agar (2% agar). R2A medium is a general medium used to plate out environmental samples taken from drinking water or aqueous environments (Reasoner and Geldreich, 1985).

5.2.2 Basalt composition

Basalt, an igneous volcanic rock, was used as analogue to the basalt rock that is found on the Mare regions of the Moon which have a low Ti content (Anand et al., 2012). The basalt was taken from the mid-ocean ridge close to the Eyjafjallajökull volcano in Iceland. The composition of this basalt is given in Table 5.1 (SCK4 in Appendix 1).

5.2.3 Flight setup

Three independent cultures of *C. metallidurans* CH34 were grown to stationary phase (OD600 nm ~ 1), cells were harvested and washed three times with 10 mM MgSO₄ and re-suspended in mineral water (10⁹ cells/ml, OD600 nm = 1). Five ml of this cell suspension was transferred to silicone cryogenic vials (VWR international, Belgium) and supplemented with or without 10 w/v% basalt, which was crushed to 1-2 mm in size, washed in deionized water and heat sterilized beforehand. A control without cells containing only water and basalt was also prepared. Two replicate sets were prepared: one for flight and one for ground control.

The prepared cryogenic vials were wrapped with parafilm to secure the caps. Active temperature loggers (Smartbuttons, ACR systems, Canada) were added to the packages as well as passive radiation sensors (thermoluminescence detectors (TLDs and optically stimulated luminescence detectors (OSLDs)) (Goossens et al., 2006; Vanhavere et al., 2008) to monitor the temperature changes and the total radiation dose cumulated over the duration of the experiment.

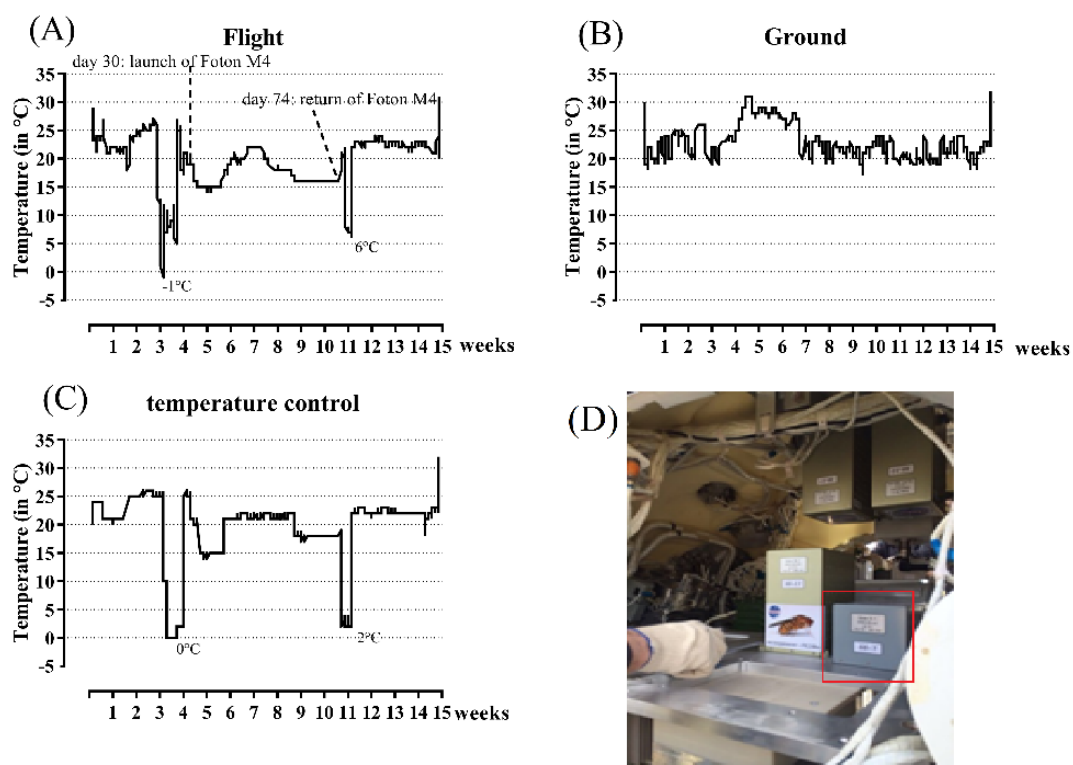


Figure 5.1: Temperature profiles measured both in flight (A) and on ground (B) during the flight experiment. The temperature profile (C) was later on simulated in order to determine the effect of these changes on the results. The experiment as set-up in the FOTON capsule is circled in red (D).

The complete flight package was kept in the dark at ambient temperature ($22.9 \pm 1.8^\circ\text{C}$, Figure 5.1A) before and during transport from SCK•CEN (Mol, Belgium) to Moscow (Russia). It left SCK•CEN on 28th of June 2014, 3 weeks before the launch of the FOTON-M4 capsule. Ten days before launch, the samples were transported from Moscow (Russia) to the launch site (Baikonour, Kazakhstan), and put in the capsule one day before launch on July 18, 2014. Samples were kept at lower temperature during this transport ($7.8^\circ\text{C} \pm 3.9^\circ\text{C}$, Figure 5.1A). Inside the FOTON M4 capsule the experiment was kept at ambient temperature ($17.6 \pm 2.5^\circ\text{C}$, Figure 5.1A). The FOTON-M4 capsule flew at 575 km altitude, with a 64.9° inclination in Low-Earth orbit and returned to Earth on September 1, 2014. Samples were returned from the landing site to Moscow at which temperatures were again low ($7^\circ\text{C} \pm 0.5^\circ\text{C}$). Samples then returned to SCK•CEN (Mol, Belgium) on September 30, 2014, while at ambient temperature ($22.4^\circ\text{C} \pm 2.1^\circ\text{C}$). The parallel ground control was kept at SCK•CEN at ambient temperature during the complete time period ($22.9^\circ\text{C} \pm 3^\circ\text{C}$, Figure 5.1B). Total radiation dose absorbed in water for the flight samples was measured and was 20.1 ± 1.47 mGy over the whole experiment duration. Within ISS, on average, the annual radiation level is 70 mGy, which is slightly lower than that measured within the flight setup (NASA, 2017). For the ground experiments the total dose was 1.2 ± 0.02 mGy, as annual background radiation accounts for 5.4 mGy, this is thus similar to what is measured within this setup (NCRP, 2015). The temperature profile (Figure 5.1C) was later on mimicked with the same setup in order to determine the effect of these changes on the results.

5.2.4 Post-flight analysis

After return to SCK•CEN, the samples were kept at ambient temperature and processed for analysis. Liquid was aspirated and 1 ml of this solution was transferred to sterile tubes for the following analyses: (1) two hundred microliters was used to estimate the number of viable cells plating a serial dilution on R2A and 284MM agar, (2) two hundred microliters was used for flow cytometry analysis, ATP and PHB measurements and (3) six hundred microliters was used to measure pH. The remaining 4 ml of the solution was used for ICP-OES analysis (see section ICP-OES, 5.2.5.).

Contamination was revealed on the counting agar plates of the flight samples both on 284MM and R2A in a level to 10^4 - 10^5 cells/ml, as colonies visually different from CH34 were present until these dilutions. The cause of this contamination is unclear. No

contaminations were found in the parallel ground control set, which was prepared and analyzed at the same time.

5.2.4.1 Sonication

After liquid aspiration, a piece of the basalt was aseptically removed from the tubes and analyzed with Scanning Electron Microscopy (see section 5.2.4.3). Next, 5 ml filtered mineral water was added and the solution was probe sonicated for 3 min at 20kHz, 4W at low-intensity to release the biofilm and intact cells from the biofilm from the basalt. The protocol for sonication was determined before the flight experiment based on previously described protocols (Kobayashi et al., 2009) and was found to be optimal at the conditions described above. After sonication, the solution was again analyzed as described in the post-flight analysis.

5.2.4.2 Flow cytometry

Samples were stained and analyzed with flow cytometry to analyze physiology and impact of space flight conditions on the CH34 cells. This was done according to the optimized procedures, described in Buysschaert et al. (2016), Van Nevel et al. (2013) and SLMB recommendation for characterizing drinking water communities (see also section 3.2.3). Cell suspensions were diluted 10,000 times in 0.2 μ m filtered Evian mineral water, as this gave the lowest background fluorescence. Next, the different dyes were added and cell suspensions were incubated at 35°C. The tested dyes include DiBAC₄(3) (Sigma Aldrich, U.S.A.), cFDA (Sigma Aldrich, U.S.A.), SYBR Green (Sigma Aldrich) and PI (Sigma Aldrich). The different dye concentrations and incubations times are shown in Table 5.1. Work solutions were all prepared in DMSO and kept at 4°C. These were prepared from stock solutions in DMSO kept at -20°C. For cFDA and DiBAC₄(3) cells were centrifuged and washed with Evian water before analyzing the samples with flow cytometry to eliminate background signals from the staining solution. For the other dyes, stained cell solutions were analyzed directly after incubation and the cells were not washed.

Stained bacterial suspensions were analyzed on an Accuri C6 (BD, Erembodegem) with a blue (488 nm, 20mW) and red (640 nm, 14.7 mW) laser which was calibrated according to the manufacturer's recommendation. Standard optical filters were used and included FL-1 (530/30 nm), FL-2 (585/40 nm) and FL-3 (670 LP) for the blue laser and FL-4 (675/25 nm) for the red laser. The dyes DiBAC₄(3), cFDA, and SYBR Green were all detected with FL-1, PI was detected on FL-3. A quality control with 6 and 8

peaks fluorescent beads (by manufacturer BD, Erembodegem) and cleaning cycle was performed prior to experiments to assess both the accuracy (bead count and position) and the cleanliness of the machine. Samples were analyzed using the Accuri C6 software (version 1.0.264.21). The flow files were uploaded to FlowCyt database (FR-FCM-ZYZQ).

The different dyes are used to test for different physiological parameters and determine the effect of the flight conditions on CH34. Sybr Green (SG) was used to stain all cells as this stain can enter both intact and damaged cells independent from their physiological state. SG enters the cells in both permeabilized and non-permeabilized cells due to its positive charge which allows it to pass it through the membrane (Veal et al., 2000;Berney et al., 2007;Berney et al., 2008). DiBAC₄(3) staining allows differentiating between cells with an intact membrane potential and normal polarization and cells which have a depolarized membrane or lost membrane function as DiBAC₄(3) can only enter the latter due to its anionic structure. Once in the cell it binds to positively charged proteins or hydrophobic regions. No binding of DiBAC₄(3) with outer membrane structures is observed. Increased depolarization of the membrane also causes more influx of DiBAC₄(3) while hyperpolarization causes a decrease in influx and thus fluorescence (Berney et al., 2008;Muller and Nebe-von-Caron, 2010;Sträuber and Müller, 2010). cFDA staining can passively enter cells and is metabolized by esterase enzymes in the cytoplasm to its fluorescent product cFluorescein (cF) that accumulates in the cytoplasm and is thus an indicator for enzymatic cell activity. Negative charges present in cF ensures that it is better retained in the cell, avoiding leakage of the product as well as reducing background signal by unspecific binding (Sträuber and Müller, 2010).

Table 5.1: Used dyes for flow cytometer analysis, incubation times and concentration

Stain	Analysis (<2000 events/ μ l)	Incubation (35°C)	Final concentration	Excitation (nm)/ Emission (nm)
SYBR Green (SG)	All cells	13 min	1x	497/520
Propidium iodide (PI)	Permeabilized cells	13 min	0.2 μ M	533/617
5(6)-Carboxyfluorescein diacetate (cFDA)	Active cells	20 min	10 μ M	488/515
Bis (1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC ₄ (3))	Membrane polarization and potential	20 min	5 μ M	490/520

5.2.4.3 SEM

To obtain an idea about cell morphology and biofilm structure Scanning Electron Microscopy (SEM) was performed. Five microliters of the solution as well as a piece of basalt was taken and transferred to a 0.2 μ m filter (Milipore) and fixed 2 times with fixation solution (3% gluteraldehyde (w/v) in 0.15M cacodylate solution, pH 7.6) in filter holders. Between each fixation step, the membrane was left to dry for 20 minutes at room temperature. Afterwards the filter surface was washed three times with the wash solution (0.15M cacodylate solution). Next, filter holders were wrapped with parafilm and stored overnight at 4 °C to let the filters dry. The next day, the filter surface was rinsed with ethanol, in ascending concentrations (30, 50, 70, 90, 95, 100 v/v%), once for each concentration. Between each step the filters were left to dry for 10 minutes. The final rinsing with 100% ethanol was done three times. To dry the surface completely, the ethanol solution was replaced with hexamethyldisilazane (HMDS), and the filters were again rinsed three times, with 10 minutes of incubation between each step. Next, the filters were air-dried in a desiccator for storage. For visualization, these filters were taped onto an aluminum stub using carbon tape and coated with gold particles using the ScanCoat machine (2x 300 milliseconds, 6-8 mbar argon; 50 mA plasma tension). They were directly thereafter visualized with the JEOL JSM6610LV SEM Microscope with a W filament.

5.2.4.4 Intracellular ATP

To measure intracellular ATP levels the BIOTHEMA intracellular ATP kit HS was used and adapted from the manufacturer's protocol for smaller volumes. Cell suspensions were diluted 1/100 and 25 µl of this dilution was added in a cuvette. Twenty five microliters of ATP eliminating agent was added and the cuvette was left to incubate for 10 min to degrade all the extracellular ATP. Twenty five microliters of cell lysis 'extractant BS' was added to the cell solution, vortexed and immediately thereafter 200 µl 'ATP reagent HS' was added. Luminescence was measured immediately after (Kikkoman Lumitester c-100). Five microliters of a 100 nmol/l ATP standard was added as an internal control and light emission (luminescence) was measured again. From the overall amount of ATP (in pmol) measured in the samples, i.e. the value I, then the average intracellular ATP concentration per cell was calculated as follows:

$$\frac{\left(\frac{I_{sample}}{I_{standard+sample} - I_{sample}} \right)}{total\ cell\ count\ (with\ SG\ in\ flow)\ in\ measured\ sample}$$

5.2.4.5 Intracellular PHB

To measure intracellular PHB levels, a Nile Red staining protocol was developed, based on the work of Degelau et al. (Degelau et al., 1995), for *Cupriavidus metallidurans* CH34 and adapted to our test conditions. Nile red binds selectively to non-polar lipid droplets inside cells and can be used to detect the presence of storage lipids (PHA/PHB) via fluorescence spectrophotometry (Greenspan and Fowler, 1985; Johnson and Spence, 2010). A Nile red working solution (200 mg/l) was prepared from a 1 g/l Nile red stock solution in DMSO, stored at -20°C. Ten microliters of the cell solution was diluted in 100 µl sterile Evian mineral water in a micro titer plate (MTP). Five microliters of the work solution was added and the MTP was incubated for 30 min at 30°C. Water without cells but with dye was used as a negative control and water without cells and without dye was used for background correction (blanco). Afterwards fluorescence of the lipid-bound Nile red was determined with a Thermo Scientific™ Fluoroskan Ascent™ Microplate Fluorometer. Nile red solution has an excitation peak at 544 nm and an emission peak at 590 nm. The normalized amount of intracellular PHB per cell was then calculated with following formula:

$$\frac{(FI_{590\ nm, 10\ \mu l\ sample} - FI_{590\ nm, negative\ control} - FI_{590\ nm, blanco}) / OD_{600\ nm}}{total\ cell\ count\ (with\ SG)\ in\ measured\ sample}$$

5.2.5 ICP-OES

At the end of the experiment, the in-organic element concentrations in the water were measured to compare changes in water chemistry induced by the rocks and cells during the experiment. Four millilitres of the supernatant from the vials without basalt fragments was taken from the tubes and centrifuged (10 000 xg; 15 min, 20°C) to pellet the cells. This supernatant was then filtered through a 0.22 µm filter to remove particles and 20 µl of 70% nitric acid was added before final inorganic element concentrations in the cell-free supernatant were determined by ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy). For concentrations below the detection limit, values were changed to zero and analyzed in the subsequent statistical analysis as such. In addition, measurements for sodium and potassium were below the detection limit.

5.2.6 Statistical analysis

For the statistical analysis of the data the GraphPad Prism (version 7.0) software package was used. For all the data of the cell suspension, normal distribution was assumed, while homogeneity of variances was tested with Levene's test and a two-tailed, one way ANOVA was used with Tukey post-hoc testing ($\alpha=0.05$).

5.3 Results

5.3.1 The effect of space flight and basalt on cell survival and physiology

5.3.1.1 Effect of space flight on culturability

To assess cell viability and culturability after space flight, a serial dilution of the cell suspensions was plated on R2A and 284MM agar (Figure 5.2A and 5.2B respectively). Culturability significantly decreased ($p<0.0001$) in the space flight experiment, compared to the initial cell number, irrespective of the presence of basalt, leading to 50% of the population remaining culturable. Statistical analysis have been summarized in Table 5.2. A greater loss of culturability was observed for the ground experiment with only 10% and 1% of the population still being culturable, with and without basalt, respectively. These results indicated that culturability decreased significantly less during space flight compared to the ground control and that the presence of basalt positively affected culturability of the ground control.

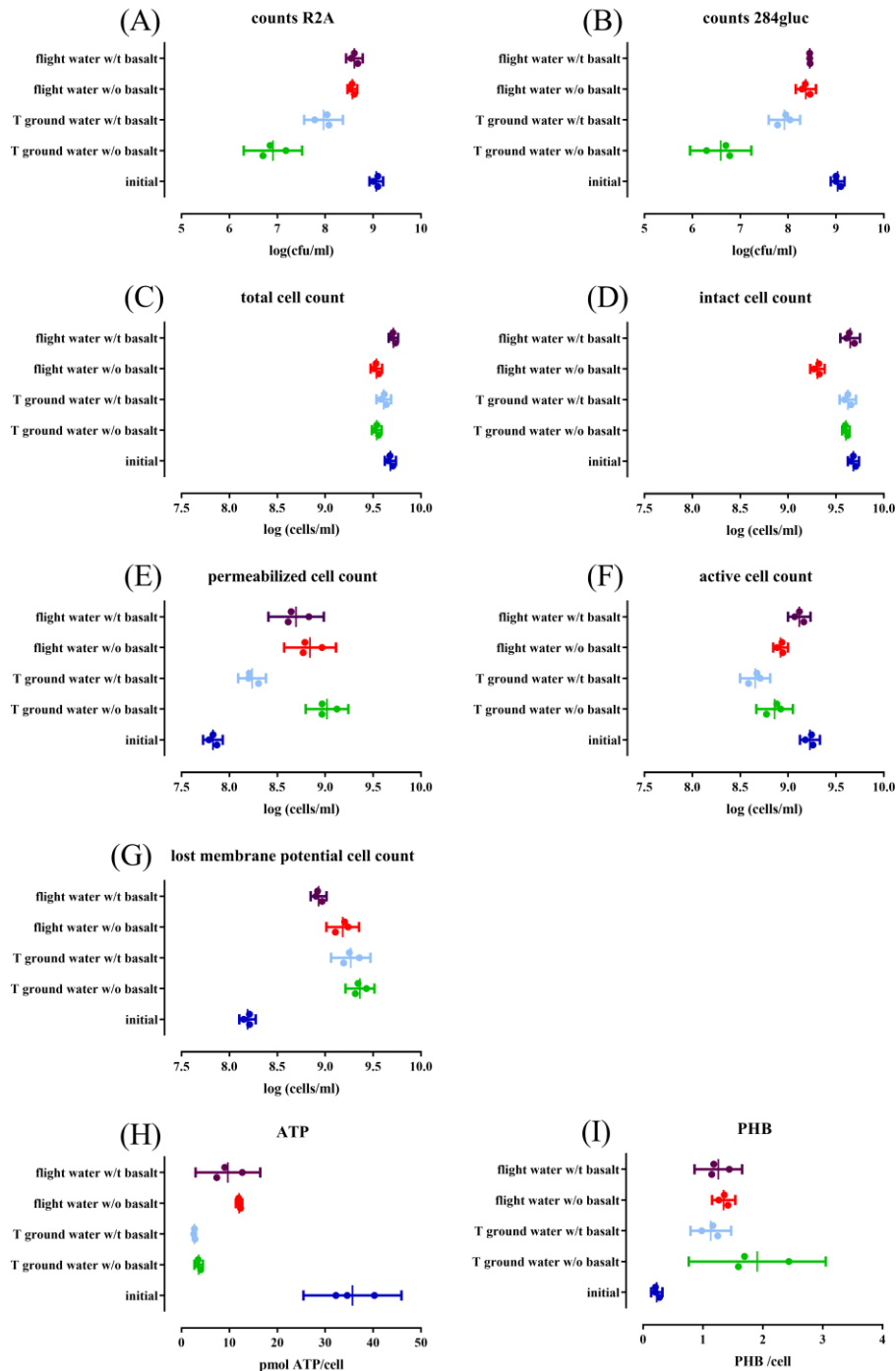


Figure 5.2: Cell physiology, culturability ATP and PHB levels of an initial stationary phase culture of CH34 cells stored in mineral water with (indicated with w/t) and without (w/o) basalt, analyzed after the three month flight experiment and after the control temperature ground experiment. All replicates are represented by a dot; the mean is indicated as a line as well as the 95% confidence interval between the brackets. Samples were plated on R2A (A) and 284 MM (B) to determine culturability of CH34. The total cells number (SG) (C) as well as the number of intact cells (SGPI) (D), permeabilized (SGPI) (E), active (cFDA) (F) and cells which have lost their membrane potential (DIBAC₄(3)) (G) were measured. ATP (H) and PHB (I) content of the cells was also measured.

5.3.1.2 Effect of space flight on physiology

The impact of space flight conditions and the presence of basalt on physiological changes within the cells were analyzed with flow cytometry by staining with specific functional dyes. The total cell numbers, measured with SYBR Green (SG), decreased slightly but significantly in samples without basalt ($p < 0.0002$) compared to the initial cell number as well as in the ground sample with basalt ($p < 0.0336$). When ground and flight samples were compared, flight samples with basalt significantly differed from the ground samples ($p < 0.003$) (Figure 5.2C). When intact cell numbers were compared (by measuring SG positive cells when stained with both SG and PI, Figure 5.2D) to the initial setup (with 99% of the population intact in the initial setup) only the flight sample without basalt differs significantly, containing 60% intact cells ($p < 0.0001$). In addition, these samples also were significantly different from the flight samples with basalt (86%) ($p < 0.0001$) and ground with and without basalt (90%; $p < 0.0001$). Permeabilized fractions (measuring the PI positive cells when stained with both SG and PI, Figure 5.2E) increased significantly in all test conditions ($p < 0.0001$) compared to the initial (contained 1% permeabilized cells). Both flight conditions with and without basalt and ground control without basalt contained more permeabilized cells than the ground with basalt, which only contained 5% permeabilized cells. The number of cells stained with DIBAC₄(3) (Figure 5.2F), related to cells that lost their membrane potential, significantly increased ($p < 0.0014$) in all conditions compared to the initial amount in the initial culture (3%). Less membrane potential-defected cells were observed in the flight samples with basalt (16%) significantly differing ($p < 0.0078$) from the ground samples and flight samples without basalt, indicating that the presence of basalt and flight conditions reduced the number of cells that lost their membrane potential. Cell activity measurements, with cFDA, (Figure 5.2G) showed that the number of active cells in the flight experiment with basalt did not significantly differ ($p > 0.1647$) from the initial setup (35%), in contrast to the other samples ($p < 0.0095$) with decreased numbers of active cells. Basalt also increased the number of active cells in the flight sample with basalt (26%) compared to other conditions.

5.3.1.3 Effect of space flight on energy status

Intracellular ATP was measured to determine the energy status of the cells (Figure 5.2H). When ATP levels were compared with the initial culture ($p < 0.0001$), ATP levels decreased 3-fold in the stored cells in water, in all conditions. Flight had a significant effect ($p < 0.0439$) on ATP levels with flight samples containing more ATP/cell than the

ground samples. Basalt did not impact ATP levels, neither for ground nor flight samples. In addition, the energy stock of the cells was estimated by measuring PHB content (Figure 5.2I). The PHB content per cell significantly increased after the three months of storage of the cells in water ($p < 0.005$), in all conditions, compared to the initial culture and on average doubling PHB concentration. No significant difference ($p > 0.0792$) was seen between ground and flight samples except for the ground samples without basalt ($p < 0.0376$) which contained more PHB than the ground and flight samples with basalt.

5.3.2 The effect of basalt and space flight on biofilm formation

5.3.2.1 SEM microscopy

SEM analysis showed biofilm formation on basalt under flight conditions and in the ground control experiment, although the biofilm formed during flight conditions was less developed (Figure 5.3). On the ground the basalt surface is completely covered with biofilm cells, while for the flight samples some basalt surface can still be seen (Figure 5.3). The level of biofilm formation in the ground control samples is comparable to that observed in preflight preparation experiments with the same setup.

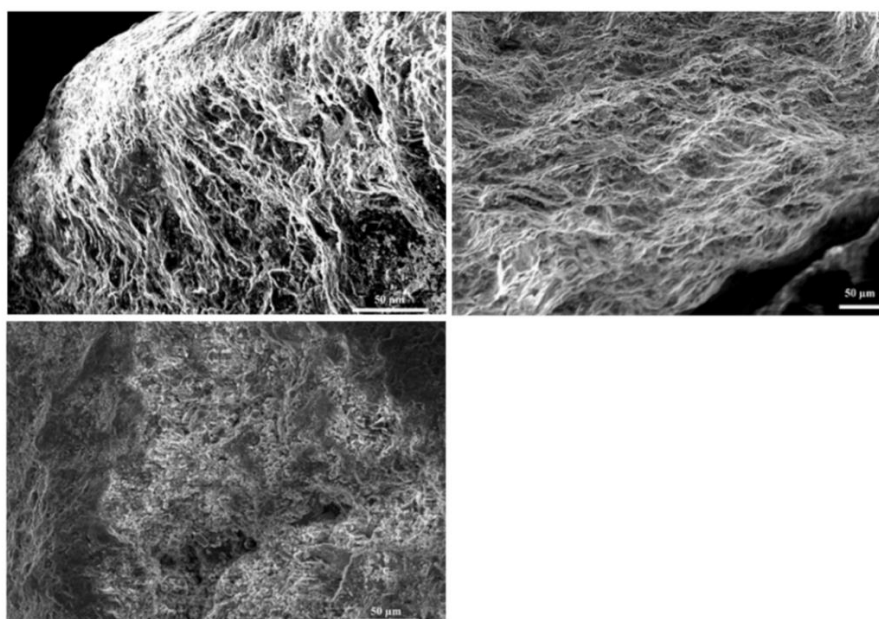


Figure 5.3: Images obtained by SEM microscopy after 3-month flight experiment. Upper left is a top SEM image of basalt from the flight experiment and upper right is a sideways SEM image of basalt from the ground experiment. The control, lower left is a top SEM picture of basalt after the 3-month experiment without cells, showing the surface of the basalt. Both in the ground and flight experiment, a biofilm covered the basalt surface. For flight, the observed biofilm was less thick and parts of the basalt surface were still exposed, for the ground experiment individual biofilm threads were not discerned and the whole basalt surface was covered.

5.3.2.2 Effect of space flight on culturability of biofilm cells

When biofilm cells were cultured on R2A and 284 MM agar (Figure 5.4A and B respectively), the viable count for flight was significantly higher ($p < 0.0058$) than the ground samples. The number of culturable cells in the biofilm fraction was also 1-1.4 log lower than in the planktonic cell fraction, indicating a significant impact ($p < 0.0003$) of the biofilm mode of growth on culturability.

5.3.2.3 Effect of space flight on physiology of biofilm cells

The total biofilm cell number was significantly lower ($p < 0.0004$; Figure 5.4C) in the flight samples compared to the ground. Flight samples also contained significantly more permeabilized cells ($p < 0.004$; Figure 5.4E) and less intact cells ($p < 0.0001$; Figure 5.4). No significant differences were observed for activity (Figure 5.4G) and membrane potential (Figure 5.4F) between flight and ground conditions.

5.3.2.4 Effect of space flight on energy status of biofilm cells

No significant difference was observed between the intracellular ATP levels nor the PHB content of biofilm cells from the flight and ground experiment (Figure 5.4H). In contrast, the ATP content of the biofilm cells is 10x lower than the ATP content ($p < 0.0006$) of the planktonic cells while the intracellular PHB content does not significantly differ from the planktonic cells in suspension (Figure 5.4I).

5.3.3 The effect of basalt on element release

ICP-OES was performed to quantify magnesium, aluminium, calcium, iron, copper and phosphate in solution and to evaluate the possible impact of CH34 cells on the leaching of elements from basalt (Figure 5.5). The long-term storage of basalt in mineral water did not significantly impact the concentration of any of the five tested elements, except for calcium. Water with basalt, both from flight and ground contained significantly less calcium compared to the start ($p < 0.0225$), indicating that basalt triggered a calcium complexation and removal from the water. The presence of CH34 cells impacted these concentrations: in the ground experiments there was more magnesium found in the water with cells with basalt compared to samples from the ground without cells with basalt ($p < 0.0275$) and the start of the experiment ($p < 0.0181$). No significant difference could be seen for magnesium in the flight samples compared to the ground samples. This is also the case for iron where samples from the ground samples contained significantly more iron compared to the condition without cells ($p < 0.0254$) and with and without basalt ($p < 0.0468$). Next, both ground and flight samples with basalt with cells

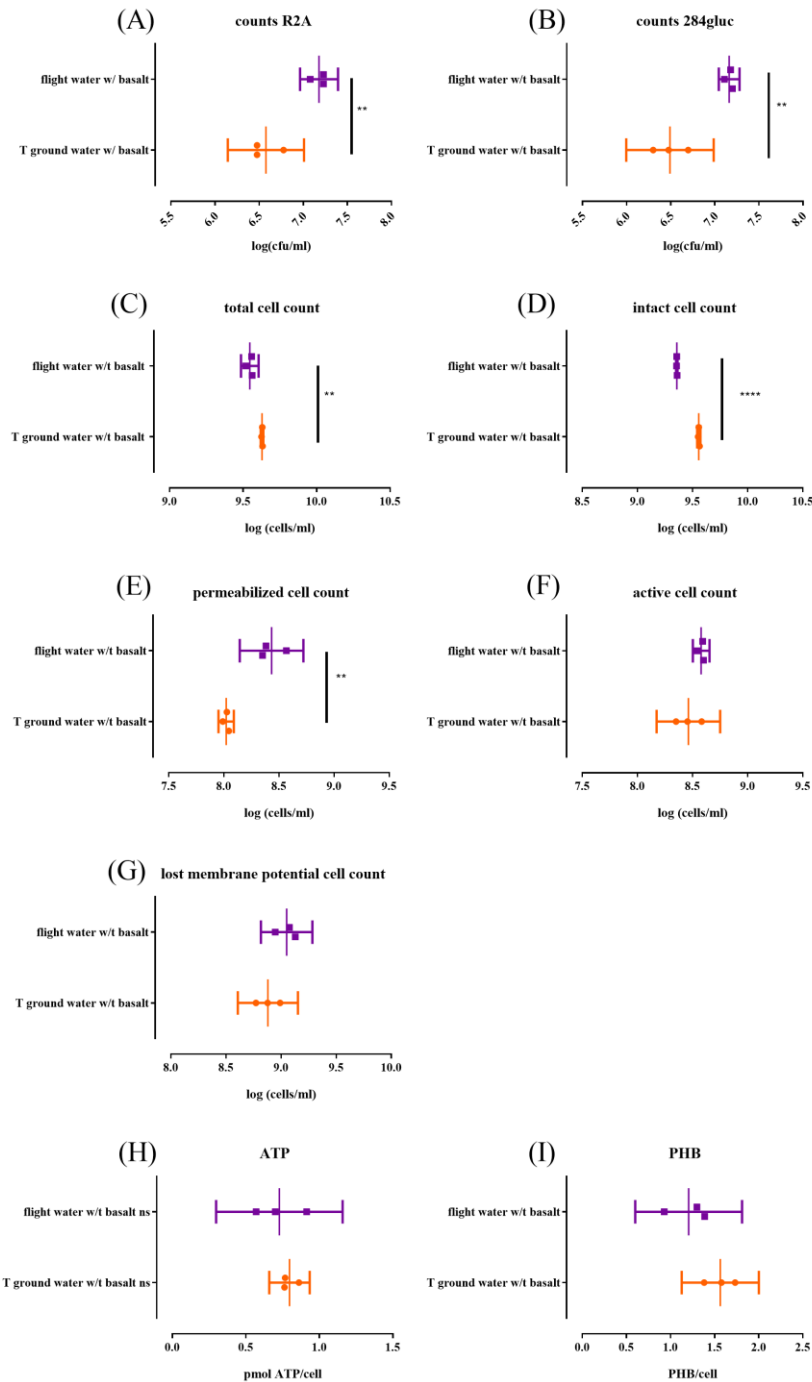


Figure 5.4: Cell physiology, culturability ATP and PHB levels of the CH34 biofilm fraction after sonication of both flight and ground samples with basalt, analyzed after the three month flight experiment. All replicates are represented by a dot; the mean is indicated as a line as well as the 95% confidence interval between brackets. For the statistical analysis, one way ANOVA was used with Tukey post testing ($\alpha=0.05$). Significances are indicated with (*) (with: ** $p<0.01$ and **** $p<0.0001$). Samples were plated on R2A (A) and 284MM agar (B) to determine culturability of CH34 biofilm cells. The total cells number (SG) (C) as well as the number of intact cells (SGPI) (D), permeabilized (SGPI) (E), active (cFDA) (G) and cells which have lost their membrane potential (DIBAC₄(3)) (F) were measured. ATP (H) and PHB (I) content of the cells was also measured.

contained significantly more copper than with basalt but without cells ($p < 0.0013$) and with cells but without basalt ($p < 0.006$). Flight and ground samples were however not significantly different from one another for copper ($p > 0.5597$). For calcium, only the cells from the flight experiment without basalt were significantly different from the other conditions ($p < 0.0251$), not showing any calcium complexation because basalt was not present in the samples. Phosphate concentrations in the ground and flight samples without basalt were significant higher than the conditions which contained basalt with ($p < 0.0004$) and without cells ($p < 0.0491$).

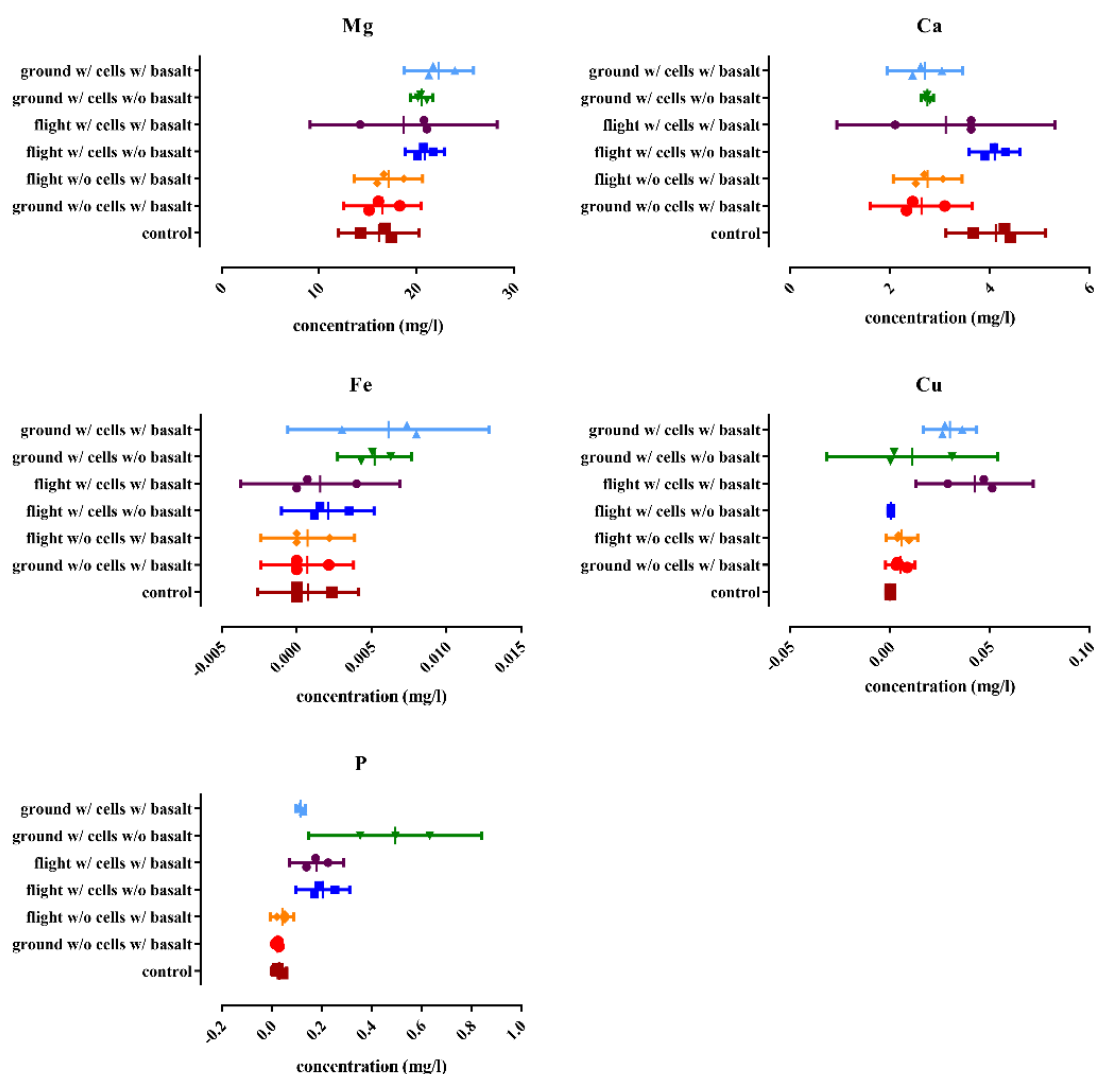


Figure 5.5: Results of the ICP-OES analysis for the magnesium (Mg), total phosphate (P), calcium (Ca), iron (Fe) and copper (Cu) content expressed as the concentration in the suspension (in mg/l). The "control" is the concentration of the respective elements in the water used at the start of the experiment. All replicates are represented by a dot, the mean is indicated as well as the 95% confidence interval.

Table 5.2: Summarizing table of the results for the different parameters of the planktonic cell fraction as well as the statistical significance (P-value), culturability decrease and percentages of the population measured with flow cytometry.

Parameter	Condition	Amount	Significance	P-value	Amount
culturability	initial	9 log =100%	ground	<0.0001	95% log decrease
	flight		flight	0.0162	50% log decrease
	ground		ground	0.0025	90% decrease
	basalt		ground	<0.0001	90% log decrease
total cell count	initial		ground	0.0336	
	flight		flight w/o basalt	0.0002	
			ground	0.0185	
			flight w/t basalt	<0.0001	
	basalt		ground	0.0247	
intact	initial	99%	flight w/o basalt	<0.0001	60%
	flight	73%	flight w/o basalt	<0.0001	60%
	basalt	86%	flight w/o basalt	<0.0001	60%
permeabilized	initial	1%	ground	0.0014	15%
			flight	<0.0001	17%
	flight	17%	ground	0.0078	15%
			flight w/t basalt	0.0006	10%
	basalt	6%	ground w/t basalt	<0.0001	4%
active	initial	35%	ground	<0.0001	16%
			flight w/o basalt	0.0003	24%
	flight	24%	ground	0.0013	16%
			flight w/t basalt	<0.0001	25%
	basalt	18%	ground	0.0073	16%
lost membrane potential			flight	0.0095	25%
	initial	3%	ground	<0.0001	56%
			flight	<0.0001	30%
	flight	30%	ground	0.0251	56%
			flight w/t basalt	0.0003	17%
ATP	basalt	27%	flight w/t basalt	0.0029	17%
	initial		flight	<0.0001	
			ground	<0.0001	
	flight		ground	0.0439	
PHB	initial		ground	0.005	
			flight	0.002	
	flight		flight w/t basalt	0.0376	
	basalt		ground w/t basalt	0.0135	

5.4 Discussion

To evaluate the effect of space flight conditions on the survival of *Cupriavidus metallidurans* CH34, a three-month space flight experiment on board the Russian FOTON-M4 capsule was conducted with cells suspended in mineral water. In addition, the effect of the presence of basalt and biofilm formation on basalt was scrutinized.

As expected, long-term storage had a significantly detrimental effect on physiology and culturability of CH34 cells. Both flight and ground samples with and without basalt had lower culturability, less ATP and more PHB per cell compared to the beginning of the experiment, and in addition contained more permeabilized cells and cells that have lost their membrane potential.

Decreased culturability, but no decline in total cell numbers, indicates that the cells surviving in these oligotrophic conditions transition into a more dormant state. This is already been shown in other experiments in similar oligotrophic conditions (Kell et al., 1998; Oliver, 2005). Both the presence of basalt and flight conditions had a positive effect, lessening the impact of the survival conditions, where 10% of the culture was culturable with basalt and 50% in flight in contrast to 1% in the ground experiment.

The energy status of these cells was also assessed by analyzing the ATP and PHB content, as in CH34 most of its stored energy is in the formation of polyhydroxybutyrate (PHB) (Sato et al., 2004; Janssen et al., 2010; Budde et al., 2011). When ATP levels were compared to the start, these were significantly lower and PHB significantly increased compared to the starting conditions. The CH34 cells thus reduce their 'immediate operational' energy levels (ATP) but increase their energy storage levels (PHB) when put in these survival conditions. Space flight conditions seem to counteract this decrease in ATP content, while basalt limited the PHB accumulation of the cells.

The drop in culturability thus coincides with a three-fold decrease in energy levels (measured ATP), and a two-fold increase in energy stock levels (PHB) over the three months flight experiment. Previous studies show that cells accumulate PHB in nutrient poor environments in mineral water (Kadouri et al., 2005). Although, not investigated here, PHB accumulation could result from metabolic redirecting of proteome or lipid cellular fractions seen in closely related *Ralstonia eutropha* H16 (Brigham et al., 2010; Sharma et al., 2016); cryptic growth as more permeabilized cells are seen in

these conditions (McAlister et al., 2002); usage of leached byproducts of the plastic tubes (Jones et al., 2003) or residual organic fractions still present on the basalt. Other factors such as exposure to stress conditions (Povolo and Casella, 2000; Rojas et al., 2011) and phosphate limitation (Shang et al., 2003; Budde et al., 2011) can trigger PHB accumulation as well. It has also been shown that PHB utilization during starvation conditions results in metabolic activity and culturability (James et al., 1999; Trevors, 2011; Najdegerami et al., 2012). PHB accumulation on the other hand can result in loss of culturability which is also seen in our results (Holmquist and Kjelleberg, 1993).

Flight conditions and basalt had a positive impact on physiology, counteracting some of the detrimental storage effects. Cells in the flight conditions with basalt contained the highest amount of total, intact and active cells while fewer cells lost their membrane potential. In addition, samples containing basalt, both on ground and in flight, contained less cells which were permeabilized or lost their cell membrane potential and had higher total and intact fractions. In summary, fewer cells lose their cell membrane potential, more are active and keep a higher ATP level and lower PHB level resulting in higher culturability. It has been seen before, in other experiments, with actively growing cells in culture medium, that indeed space flight can have a significant impact on physiology, predominately due to microgravity effects. It was shown that bacteria have increased metabolic activity, higher biomass, and produce more secondary metabolites in these conditions allowing growth (Leys et al., 2004; Nickerson et al., 2004; Mastroleo et al., 2009; Taylor, 2015). For cells tested in survival conditions, *Ralstonia pickettii*, starting from a 10^5 cells/ml in water, showed a higher cell break down and autolysis rate in simulated microgravity compared to normal gravity after 14 days in this survival setup (Baker and Leff, 2004).

Space flight conditions have an impact on the bacterial cell physiology, which in turn can have an impact on microbial rock weathering and biofilm formation to maximize survival. Biofilm formation was different in flight and ground conditions, observed using SEM pictures. A clear biofilm was formed in ground conditions but this is not the case in the flight experiment in which the biofilm was shown to be less developed and differently structured. When total cell numbers were determined there was also a significant difference between ground and flight samples indicating that in flight fewer cells were present in the biofilm. This contrary to some papers where biofilm formation increased in space flight conditions (McLean et al., 2001). Cells present in the biofilm

during flight also show different physiology traits, as more were permeabilized, less intact, but more culturable. In biofilm cells, ATP content or PHB content did not differ between ground and flight samples. Flight thus impacts the number of cells which transition into the biofilm state which increases culturability but more cells are permeabilized and less are intact.

For the elemental analysis, basalt had an effect on calcium removal from the water, irrespectively of the presence of cells and both in ground and flight experiments, probably due to complexation on basalt (Stockmann et al., 2011). Also when adding cells, calcium is removed from the water even in samples without basalt, indicating that cells can take up calcium in this condition. This was also observed for phosphate as concentrations were lower, both in ground and flight experiments, with basalt, while without basalt more phosphate was remaining in suspension. Cells released more magnesium and iron with and without basalt in the ground samples while water from the flight experiment contained less of these elements, showing that cells keep more of these elements intracellularly in these conditions, irrespectively of the presence of basalt. Copper is released from the basalt in the presence of cells both in flight and ground, thus cells having a positive impact on copper release from basalt.

Our results indicate that in general flight conditions as well as basalt had a positive effect on survival, counteracting some of the detrimental effects of inoculum storage in water. Cells were more culturable, contained more ATP and more cells were present which were intact and active while fewer cells lost their membrane potential. This changes cellular physiology. Cells will thereby not as easily transition into a more 'dormant' state and start forming a biofilm on basalt. With this experiment, we provide for the first time results for the combined effect of space flight conditions and the presence of basalt on survival in water. We could also show that in space cells form slightly less biofilm and so that there is an impact of space flight conditions on microbe-mineral interactions. As these experiments were performed with limited amount of samples and ground and flight samples were prepared separately, batch specific changes may have occurred. It is clear however that this preliminary experiment is only the very first step and much more is still to be tested and learned. Results presented here are preliminary results and more (long lasting) experiments are needed to draw definite conclusions. Nevertheless, this research may hopefully open the door for

future studies and potentially applications of microbe-mineral interactions in space and even on Earth.

5.5 Acknowledgements

This work was supported by the European Space Agency (ESA-PRODEX), Belgian Science Policy (BELSPO) through the E-GEM/BioRock project (Bo Byloos) and the Inter-University Attraction Pole (IUAP) “ μ -manager” funded by the Belgian Science Policy (BE, 305 P7/25). We thank Kai Finster and Charles Cockell for the coordination of the BIOROCK project, and Vyacheslav Ilyin for incorporating biomaterial within Russian spaceflight experiment “MIKROB”. The authors would also like to thank the people of the microbiology unit at SCK-CEN and the IMBP institute for all the support and help during pre-and post-flight analysis and preparations.

Chapter 5

Chapter 6

The use of flow cytometry to assess microbial viability

The use of flow cytometry to assess microbial viability

Abstract

Flow cytometry has been proposed as a rapid and quantitative method to determine bacterial viability. Although different stains can be used to establish viability, staining protocols are inconsistent and lack a general optimization approach. We therefore evaluated protocols for both functional stains and red-excitable DNA stains. New protocols for both the red-excitable DNA dyes SYTO 59 to 64 and SYTO 17 as well as new combinations with functional dyes for multicolor flow cytometry were developed. The functional dyes tested were carboxyfluorescein (cFDA), Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DIBAC₄(3)), dihydroethidium (HE), 5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester (cFDA-SE) and Nile red (NR). Staining performance was assessed for a Gram-positive and Gram-negative bacterium, *Lactobacillus brevis* LMG 18022 and *Cupriavidus metallidurans* CH34, respectively. Continuous measurements and time point measurements were performed to assess the temporal variability, the optimal stain concentration and the effect of EDTA as stain enhancer. Results showed that both stain concentration and membrane composition influence dye uptake, and that for certain stains the addition of EDTA is necessary for an optimal performance. We demonstrated the effectiveness of optimized double and triple staining protocols and showed that different physiological parameters of the cell population can be assessed simultaneously.

Chapter partially redrafted after: Buysschaert, B. and Byloos, B. Leys, N., Van Houdt, R. and Boon, N. (2016). Reevaluating multicolor flow cytometry to assess microbial viability. *Applied microbiology and biotechnology*. 100(21):9037-9051.

6.1 Introduction

Flow cytometers, invented in the late 1940s, found their way into microbiology in the 1980s because of the technological advances (Wang et al., 2010). The main advantage is the speed of single cell analysis and consequently the possibility to make a detailed analysis of the cell population and its heterogeneity. This information can be used to assess microbial viability and survival in different environments. In this respect, information about the heterogeneity within bacterial populations can improve our understanding of microbial ecology, to expand our knowledge and reduce system failures in the area of microbial technologies. A second advantage is the possibility to simultaneously assess the physiological state and growth behavior enabling control of metabolic processes both online and offline (Breeuwer and Abee, 2000a; Rieseberg et al., 2001; Muller, 2007; Shi et al., 2007; Boi et al., 2015; Lieder et al., 2016).

Multicolor flow cytometry is a good approach to simultaneously estimate and assess multiple features and to characterize the heterogeneity of a community in detail. However, staining bacteria is a complex interplay between dye chemistry, the target organisms and the staining conditions. For microbiological applications, the diversity of bacterial species is challenging, as even closely related organisms are known to behave very differently, making it difficult to analyze bacteria in a standardized way (Shapiro, 2000). Hence, it is important to have a reasonable amount of standardization in terms of stain concentration, used buffers, incubation time, need for permeabilization or fixation, and the necessary controls in order to compare different samples. In addition, a better understanding of the staining chemistry is important to estimate the reliability of a staining protocol for a specific research setup. Unfortunately, it is exactly on those aspects that many available studies lack the necessary information and where data are poor and inconsistent. An overview of the parameters important for protocol optimization are given by Hammes et al. (2011). Very few 'true' multicolor protocols, where dyes are combined in one sample, have been developed in such a way for microbiological applications. In this mini-review, we will discuss the discrepancy between protocols for some popular stains and offer a way in which staining could be optimized for a specific setup. The number of available dyes is vast and summarizing them all in detail is beyond the scope of this mini-review as not all dyes are useful to determine viability. For more information consult Hammes et al. (2011), (Sträuber and Müller, 2010), Tracy et al. (2010) and Shapiro (2003). Furthermore, we included

original data using red-excitable SYTO dyes combined with functional stains for double and triple staining applications.

6.2 Viability

The concept of viability has been introduced more than 100 years ago and was based on the capability of bacteria to grow on agar plates, a method which is still used today. However, it is now clear that only a small fraction of bacteria can grow on classic media and that they can switch between different states of persistence, culturability and dormancy showing variances in size, shape or nutritional behavior (Hammes et al., 2011). In a natural habitat, bacteria can have even more different physiological states depending on their metabolic activity, which can vary within spatial and temporal distributions. Bacterial abundance, activity and community composition also changes in response to abiotic and biotic stresses and significant differences can occur over minutes, hours or weeks. Specific bacterial activities, contributing to the overall function of the community, need to be scaled to the number of bacteria participating in that activity in order to get a full understanding of microbial ecological behavior and viability.

Although, numerous studies indicate this unculturability of bacteria, some advances have been made. Recovery of the unculturable bacteria has been difficult to prove, produce and reproduce (Lopez-Amoros et al., 1995; Gasol and Del Giorgio, 2000; Amor et al., 2002a; Falcioni et al., 2008; Hammes et al., 2011; Boi et al., 2015). Hence, the phenotype and physiological basis for these processes have not been defined and is still open to investigation (Kell et al., 1998; Breeuwer and Abee, 2000a; Epstein, 2013). A good review for the range of distinct bacterial states, the viable but non culturable phenomena, and the difference in dormancy is given by Kell et al. (1998) and Li et al. (2014). For more information about unculturable bacteria, we recommend the detailed review of Stewart (2012).

Defining viability is thus challenging and it requires an assessment of various cellular criteria (such as growth, DNA transcription and RNA translation, energy generation, metabolism) that constitute or relate to life and death of an organism. Examples of parameters, used to measure these criteria, are; membrane integrity, membrane potential, pH gradient, pump activity, respiration, cell morphology, enzyme activity and reproduction. The most important function herein is maintaining the membrane

potential, as bacteria need to be able to maintain an intracellular environment that supports energy generation (Breeuwer and Abee, 2000a; Hammes et al., 2011).

Therefore, to fully comprehend viability, tools that accurately and reliably measure these parameters need to be established (Boi et al., 2015). As mentioned, the golden standard that is used to measure viability is culturing, which is cheap and easy but a slow, retrospective and a binary tool. Other conventional methods, such as fluorometric, luminometric or conductivity assays, have also been established to identify and characterize bacteria in a high-throughput manner. They offer a fast and simple bulk assessment of the viability of a bacterial culture. Therefore, estimating differentiation and heterogeneity within a population in relation to viability requires a combination of single cell techniques such as cytometry, molecular characterization or microscopy (Hastings and Wilson, 1976; Ericsson et al., 2000; Jameson et al., 2003; Cangelosi and Meschke, 2014). In this respect, flow cytometry is an excellent technique as it offers single cell analysis of various parameters in a high-throughput manner with improved speed and sensitivity. Basic cell functions such as metabolic activity, respiration and membrane integrity can easily be determined simultaneously. These multiparametric data allow estimation of population heterogeneity and distinction of different viable stages. However, these data do not offer absolute discrimination between live and dead bacteria as these parameters can vary in response to different environmental factors. In addition, many methods lack standardization, thereby, creating significant variation in interpretation and estimation of viability (Roszak and Colwell, 1987; Neidhardt et al., 1990; Nebe-von-Caron et al., 2000; Prescott et al., 2004; Davis, 2014). Other emerging technological developments such as single-chip analysis, gel microdroplets, microfluidics, etc. could also improve our understanding of bacterial heterogeneity and viability. As these techniques offer single cell analysis in a controlled micro-environment, they facilitate studying the effect of certain stressors on different cellular levels simultaneously (Amor et al., 2002a; Keer and Birch, 2003; Benoit et al., 2010; Hammes et al., 2011). The multitudes of techniques mentioned above have both advantages and disadvantages for assessing viability (summarized in Table 6.1). In addition, by allowing the determination of complex physiological parameters, it has become more complex to determine 'viability' and as a consequence the 'viable' paradigm has shifted (Nebe-von-Caron et al., 2000; Falcioni

et al., 2008; Hammes et al., 2011). In general, no single technique or stain is suited for viability measurements of all species and under all conditions (Davey, 2011).

6.3 Dyes

A wide variety of fluorescent dyes are available for flow cytometry. Thus, very different aspects of microbial physiology can be assessed and monitored. However, it is important to understand how different dyes function and how they can be applied in order to draw the correct conclusions. In this mini-review we will separately discuss cell-permeant nucleic acid stains and functional stains that give information about bacterial physiology.

6.3.1 Cell-permeant nucleic acid dyes

Nucleic acids carry the genetic information used in the development and functioning of all living organisms and viruses and could therefore be a very logical indicator of life. However, it's important to note that DNA can be persistent and that dead cells may still contain DNA. For example, when bacteria are killed by UV-C irradiation, lethal thymine dimers are produced that will be stained by most nucleic acid dyes (Hammes et al., 2011). Nucleic acid stains can also act as good counterstains for labeling all organisms which are present, live or dead. Generally, nucleic acid stains can be divided in cell-permeant and cell-impermeant dyes depending on their ability to pass through the cell membrane. The latter, being unable to cross intact membranes, can therefore be considered as viability-dependent dyes. Furthermore, the difference between Gram-positive and Gram-negative bacterial cell wall structure poses additional difficulties (Shapiro, 2003; Berney et al., 2007; Sträuber and Müller, 2010) and permeabilization of the outer membrane of Gram-negative bacteria is often necessary in order to optimize staining. Either EDTA or citrate can be used for this purpose (Marie et al., 1996). Both compounds permeabilize the outer membrane by chelating cations and stripping the LPS layer of the outer membrane (Chen et al., 2004). These cations can also be deleterious as they decrease binding efficiency of certain dyes such as DAPI or Hoechst 33342 (Marie et al., 1996). Here, we will focus on EDTA as previous results from Marie et al. (1996) showed that EDTA gave similar results to those with citrate.

Table 6.1: Overview of advantages and disadvantages of the different viability techniques

Method	Cellular function	Execution time	Specificity	Automation	Time to result	Challenges
Plate count	Reproduction	+++	+++	No	++++	Appropriate conditions are necessary to distinguish cells which can grow
Microscopy	Cell morphology	++	+++	No	+	Difficult to optimize
Fluorescent/ Illuminoteric measurments	Reproduction Enzym activity	+	+	Yes	+	Bulk assessment, no impression of heterogeneity
ATP measurments	Energy status	+	+	Yes	+	Bulk assessment, no impression of heterogeneity
Flow Cytometry	Membrane integrity, Enzyme activity, pH gradient, Pump activity, DNA and RNA intactness	++	++	Yes	+	Stain optimization discussed further, minimal 10^3 - 10^4 cells/ml necessary in order to measure accurately
Labeled substrate uptake	Membrane potential, integrity and pH gradient	+++	+++	No	++	Labeling is necessary and difficult as it is a sensitive method
RNA aptamer conjugations	RNA and DNA intactness and transcription	+++	+++	No	++	Retrospective and sensitive method
Molecular viability testing PCR /viability PCR /RT-PCR/	RNA and DNA intactness	+++	+++	No	++	Retrospective and sensitive method
Single-chip/ microfluidics systems	Membrane integrity, Enzyme activity, pH gradient, Pump activity, DNA and RNA intactness, Cell morphology	+++	+++	No	+	Expensive technique and optimization necessary

Many dyes can be used to stain nucleic acids and well-known examples are DAPI (stains DNA), and SYBR green I (stains DNA) and SYTO 9 (stain DNA and RNA). DAPI is excited by a UV laser (355 nm) but despite the good results, UV lasers are expensive and not available in the standard configuration of most bench-top flow cytometers. SYBR green I and SYTO 9, which are compatible with almost all bench top flow cytometers (ex./em. 488/530nm), have already been extensively studied and are good candidates for nucleic acid staining (Lebaron et al., 1998; Zipper et al., 2004; Berney et al., 2007; SLMB, 2012; Van Nevel et al., 2013). Besides SYTO 9, many other SYTO dyes are available. All SYTO dyes share some common characteristics such as high signal to background fluorescence, high molar absorptivity, permeant to nearly all cell membranes and high quantum yields when bound to nucleic acids (Johnson and Spence, 2010). The SYTO stains differ in their affinity to bind DNA and RNA and in their spectral characteristics, making the SYTO family a versatile tool for multicolor flow cytometry. In contrast to other DNA stains, some SYTO dyes are excited by a red laser (640 nm), increasing the possibility for multicolor combinations and enabling their use as a counterstain. Red lasers are frequently used for the popular fluorochromes APC, Alexa Fluor® 700 or APC-eFluor® 780 in combination with antibodies for research with mammalian cells. Therefore, commercial benchtop flow cytometers are often standardly equipped with red lasers. However, only a few applications are known for this laser in microbiology such as DRAQ5 as cell-permeant nucleic acid stain. Since it has been reported that DRAQ5 alters morphology and decreases cell viability, it is less suitable in combination with viability stains (Silva et al., 2010).

To further scrutinize the usability of SYTO dyes, we investigated the possible application of the red-excitable SYTO dyes 17, 59, 60, 61, 62, 63 and 64 for both the Gram-negative bacterium *Cupriavidus metallidurans* CH34 and the Gram-positive bacterium *Lactobacillus brevis* LMG 18022 (Appendix 2). First, we tested the staining kinetics by measuring the change in fluorescence over time. Results indicated that dye uptake was immediate for the Gram-positive bacterium while an incubation period of approximately 15 minutes was required for the Gram-negative bacterium to reach maximum fluorescence intensity. For the Gram-positive population, fluorescence intensities were stable, indicating time-independent uptake. For the Gram-negative population, fluorescence intensities increased over time showing time-dependent uptake. After uptake, the fluorescence signal remained stable during the entire

measurement period (30 minutes). This difference in uptake between Gram-positive and Gram-negative bacteria is likely due to differences in cell membrane composition and is also observed for blue-excitable SYTO dyes (Lebaron et al., 1998). A second test was performed where different dye concentrations and the effect of EDTA were assessed for all dyes. This test was performed because the amount of cells counted (of a known cell concentration) can vary depending on the used dye concentrations and incubation time regardless of the fluorescence intensity of the cell population. Thus, different dye concentrations and the effect of EDTA were assessed at different time points during incubation (0 min, 15 min and 30 min) for all red-excitable SYTO dyes. As a benchmark, cell counts were compared to the standard SYBR green I staining as described in the Swiss protocol for detection of bacteria in drinking water (SLMB, 2012). Results of this experiment for SYTO 60 and the SYBR green benchmark are shown in Figure 6.1. A stain was assessed as good, if cell counts did not deviate more than 10% from the SYBR green benchmark. Our results showed that a final stain concentration of 0.5 μM is preferable for all red-excitable SYTO dyes and that an incubation period between 15 to 30 minutes depending on the organism is sufficient to obtain a reliable estimate of cell numbers (Figure 6.1). Only SYTO 64 was unable to stain bacteria under these conditions, as no cells could be detected with the flow cytometer. This optimal stain concentration is comparable to that found by Comas and Vives-Rego (1997a) who tested SYTO 17 at 1 μM on *Escherichia coli*. However, our protocol relied on a shorter incubation time of 15 minutes instead of 60 minutes. This difference in incubation temperature could facilitate dye intrusion by diffusion (Johnson and Spence, 2010). As cell concentrations remained stable after the minimal incubation time it indicates that the dyes are not pumped out of the cells. In case no bleaching occurs, longer incubation times can also be used. The addition of 5 μM EDTA did not improve staining efficiency nor signal intensity, except when lower dye concentrations (0.05 μM) were used for the Gram-negative bacterium.

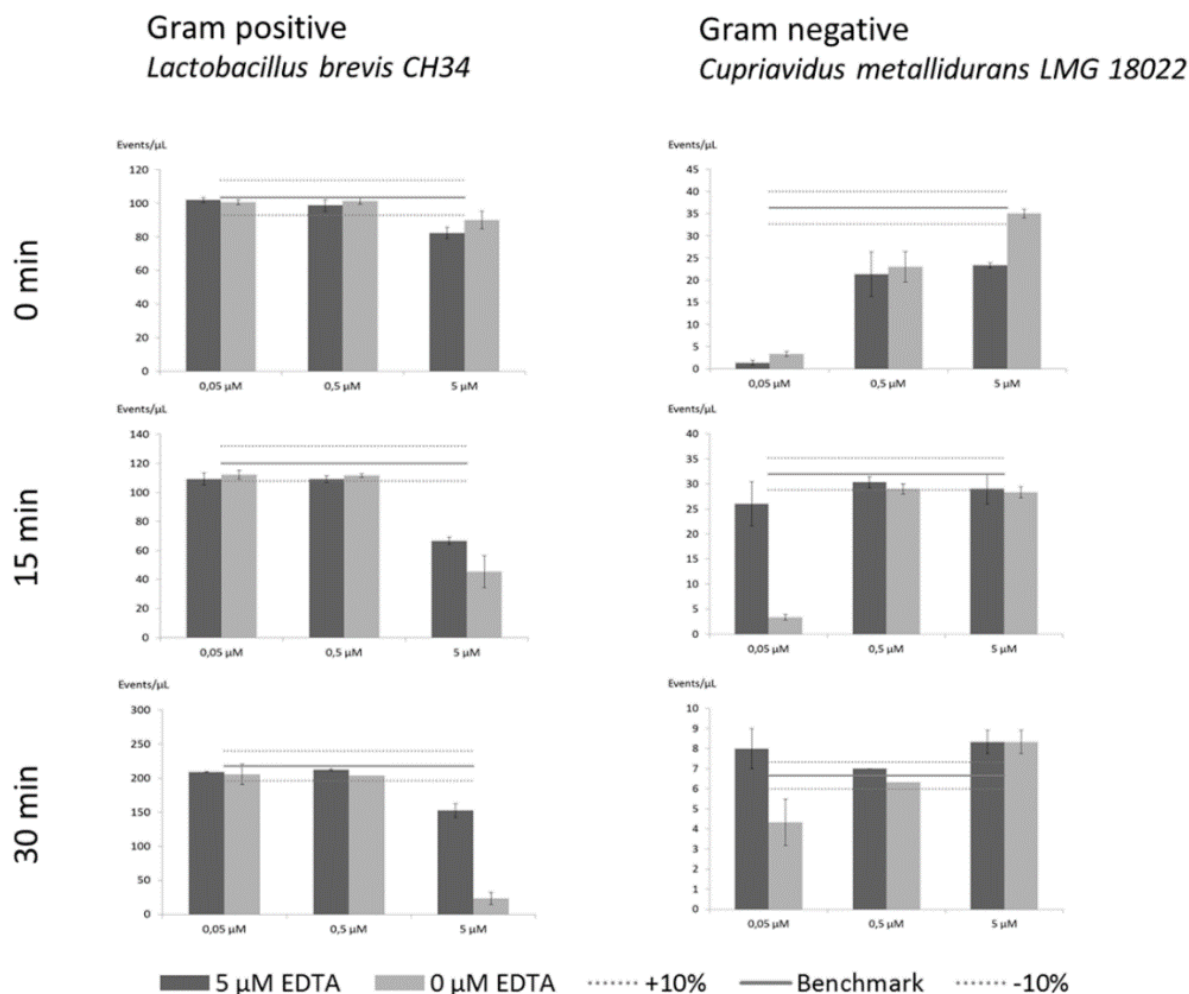


Figure 6.1: Optimization of the red-excitable dye SYTO 60 for both the Gram positive *Lactobacillus brevis* LMG 18022 (left) and Gram negative *Cupriavidus metallidurans* CH34 (right) on 3 different time points: 0 min (top), 15 min (middle) and 30 min (bottom). Staining was performed with 3 different stain concentrations (5 μM , 0.5 μM and 0.05 μM) with and without 5 μM EDTA. All samples were measured in triplicate. Cell counts are expressed as events/ μL and should be compared to the results obtained with SYBR green I staining as benchmark. A maximum 10% deviation on the SYBR green I results was accepted.

6.3.2 Functional dyes

Cell-impermeant nucleic acid stains such as propidium iodides (PI) are not able to cross membranes because of their size and charge, and are therefore used as an indicator of membrane permeabilization (Berney et al., 2007). Since membrane integrity is vital to keep the intracellular environment stable, membrane damage can be an indication of cell death (Hammes et al., 2011). However, it is known that contact time, incubation temperature and stain concentration are crucial factors for proper staining, which emphasizes the importance of standardization (Hammes et al.,

2012;Van Nevel et al., 2013). Furthermore, a recent study on *E. coli* suggested that porins and periplasmic transporters induced by substrate limitation facilitate PI entry into cells and that staining efficiency is influenced by the physiological state (Broгнаux et al., 2014). Shi et al. (2007) showed that more bacteria were stained during early exponential phase than during the early lag phase. This indicates that PI cannot be used as viability estimator and suggests the necessity for multiparameter viability determination. Besides PI, other dyes with similar mode of action are available such as SYTOX dyes, the TOTO and TO-PRO family of dyes (Shapiro, 2003).

Another aspect of viability is maintaining the cell's membrane potential. All active microbial cells need to keep their membrane potential, which is produced through a functional electron transport chain. The membrane potential also powers processes such as ATP synthesis and solute-ion transport. If the membrane potential decreases, the cell will be unable to transport essential molecules eventually leading to cell death. Membrane potential should be considered as a more conservative measurement of viability compared to membrane permeabilization because of the link between membrane potential and cell respiration (Hammes et al., 2011). DiBAC₄(3), also known as bis oxonol (BOX), is mostly used to evaluate membrane potential. It enters depolarized cells, because of its anionic structure and non-specifically binds to intracellular proteins (Muller and Nebe-von-Caron, 2010). In contrast, the cationic Rhodamine 123 (Rh123) only accumulates in cells with active membrane potential (Diaper et al., 1992). Since this stain can be actively pumped out by certain cells it has a limited use in standardized protocols (Tracy et al., 2010). Alternative dyes are 3,3'-dihexyloxacarbocyanine iodide (Di-OC6(3)), 3,30-diethyloxacarbocyanine (Di-OC2(3)) and 3,30-dipropylthiadicarbocyanine (DiSC3(5)) (Shapiro, 2003). Various protocols exist for the use of DiBAC₄(3) with final stain concentrations, incubation time and temperatures ranging from 0.24 μ M to 29 μ M (Herrera et al., 2002;Nielsen et al., 2009), 2 to 20 minutes (Lopez-Amoros et al., 1995;Comas and Vives-Rego, 1997a;Rezaeinejad and Ivanov, 2011), and room temperature to 40°C (Rezaeinejad and Ivanov, 2011;Linhova et al., 2012), respectively. Table 6.2 provides an overview of staining protocols and their references.

Table 6.2: Overview of concentration, incubation time and temperature used in staining protocols for different dyes and organisms compared with our findings.

	Concentration [μM]	Incubation Time [min]	Incubation Temperature [°C]	Organism studied	Reference
DiBAC₄(3)	5	22-40	37	<i>C. metallidurans</i> CH34, <i>L. brevis</i> LMG 18022	This study
	1	4	37	<i>Bifidobacterium adolescentis</i>	Amor et al. (2002a)
	1	2	RT ^a	<i>E. coli</i>	Comas and Vives-Rego (1997a)
	19.4	10	RT	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	Jepras et al. (1995)
	0.24	10	RT	<i>P. fluorescens</i> , <i>Pythium ultimum</i> , <i>Rhizoctonia solani</i>	Nielsen et al. (2009)
	0.5	15	37	<i>S. macedonicus</i>	Papadimitriou et al. (2007)
	1	20	37	<i>E. coli</i>	Rezaeinejad and Ivanov (2011)
	0.48	20	40	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Rault et al. (2008)
	1	2	RT	<i>E. coli</i> , <i>S. typhimurium</i>	Lopez-Amoros et al. (1995)
	1.94	7	RT	<i>C. pasteurianum</i> , <i>C. beijerinckii</i>	Linhova et al. (2012)
	29	10	RT	<i>E. coli</i>	Herrera et al. (2002)
	10	10	20	<i>E. coli</i>	Berney et al. (2009)
	3.87	10	RT	<i>Pichia pastoris</i>	Hyka et al. (2010)
cFDA	10	30-40	37	<i>C. metallidurans</i> CH34, <i>L. brevis</i> LMG 18022	This study
	50	10	30	<i>Lactobacillus plantarum</i>	Bunthof and Abee (2002)
	50	10	30	<i>Lactococcus lactis</i>	Bunthof et al. (1999b)
	10	10/20 and 30	RT/30/40	Lake water bacteria	Porter et al. (1995)
	10	10	37	Lactic acid bacteria	Chen et al. (2012b)
	10	30	37	<i>Bifidobacterium adolescentis</i>	Amor et al. (2002a)
	0.000217	10	40	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Rault et al. (2008)
	0.00217	10	40	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Rault et al. (2009)
	25	45	37	<i>S. macedonicus</i>	Papadimitriou et al. (2007)
	10.8	10	37	<i>Pichia pastoris</i>	Hyka et al. (2010)
	50	1 h	30	<i>B. cereus</i> endospores	Cronin and Wilkinson (2008a)
	10	30	37	activated sludge bacteria	Forster et al. (2002b)
	21,7	10	RT	<i>C. pasteurianum</i> , <i>C. beijerinckii</i>	Linhova et al. (2012)

	10	15	35	<i>Aeromonas hydrophila</i> , <i>B. subtilis</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. epidermidis</i> and bacteria from environmental waters	Hoefel et al. (2003)
	21.7	30	37	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	Jepras et al. (1995)
	5	30	30	<i>L. lactis</i>	Hansen et al. (2015)
cFDA-SE	0.2	38-40	37	<i>C. metallidurans</i> CH34, <i>L. brevis</i> LMG 18022	This study
	0.0448	10	40	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Rault et al. (2008)
	100	150	25-37	Strains from aquatic environment	Fuller et al. (2000)
	10	30	35	<i>Aeromonas hydrophila</i> , <i>B. subtilis</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. epidermidis</i> and bacteria from environmental waters	Hoefel et al. (2003)
	11	20	37	<i>S. pyogenes</i>	Hytonen et al. (2006)
	1	15	30	<i>Bifodobacterium</i> , <i>Lactobacillus</i>	Lahtinen et al. (2006)
	50	20	37	<i>L. casei</i>	Lee et al. (2004)
	10	30	37	<i>B. licheniformis</i>	Hornbaek et al. (2002)
	35	10	RT	<i>Lactobacillus plantarum</i>	Fitzgerald et al. (2004)
	1	10	30	<i>L. lactis</i>	Breeuwer et al. (1996)
	44.8	10	40	<i>Oenococcus oeni</i>	Bouix and Ghorbal (2015)
	50	30	30	<i>L. lactis</i>	Hansen et al. (2015)
	0.5	4	RT	<i>Cronobacter</i> spp.	Arku et al. (2011)
HE	5	40	37	<i>C. metallidurans</i> CH34, <i>L. brevis</i> LMG 18022	This study
	31.7	10	RT	<i>E. coli</i>	Herrera et al. (2002)
	10	0	RT	<i>Cupriavidus</i> , <i>shewanella</i> , <i>E. coli</i> , <i>deinococcus</i>	Baatout et al. (2005)
	0.07	4	RT	<i>Cronobacter</i> spp.	Arku et al. (2011)
Nile Red	0.13	10-40	37	<i>C. metallidurans</i> CH34, <i>L. brevis</i> LMG 18022	This study
	10 and 100	30	RT	<i>Synechocystis</i> spp, <i>E. coli</i>	Tyo et al. (2006)
	157	10-15	RT	<i>P. auroginosa</i>	Vidal-Mas et al. (2001)
	3.1	30	RT	<i>Escherichia coli</i>	Herrera et al. (2002)
	94.2	10	RT	<i>Ralstonia eutropha</i>	Gorenflo et al. (1999)
	60	10	RT	environmental bacteria	Koch et al. (2013)
	0.1258	30	RT	<i>Methylobacterium rhodesianum</i>	Ackermann et al. (1995)

Besides membranes, other aspects of the cell can be used to assess viability or functionality. All bacteria possess housekeeping enzymes such as esterases or dehydrogenases that are linked to the respiratory activity of metabolically active cells. The inactivity of these enzymes indicates metabolic inactivity, but not necessarily cell death as these enzymes can still be active even after cell death has occurred. Measurement of cellular enzymatic activity is useful although the mentioned limitations need to be considered. Generally, dyes used to monitor enzymatic activity are cleaved upon uptake in the cell, leading to the production of a fluorescent signal (Shapiro, 2003). Again, a wide variety of dyes that target different enzymatic activities are available. A popular dye is 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which is reduced by dehydrogenases to fluorescent membrane-impermeant formazan (Lopez-Amoros et al., 1997). Since respiratory activity is linked to the maintenance of the membrane potential, CTC reduction and DiBAC₄(3) diffusion are complementary (Hammes et al., 2011). Fluorescein diacetate (FDA), another popular dye, is cleaved by esterases to release the fluorescent fluorescein. Since fluorescein easily leaks from cells, FDA modifications have been developed such as carboxyfluorescein diacetate (cFDA), with better retention kinetics, and modifications of cFDA in order to further reduce leakage such as carboxyfluorescein diacetate acetoxymethyl ester (cFDA-AM), 20,70-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-AM (BCECF-AM), calcein-AM and carboxyfluorescein diacetate succinimidyl ester (cFDA-SE). Evidently to avoid leakage, cell permeabilization (e.g. by EDTA or citrate) to improve staining is not recommended with those dyes. Furthermore, permeabilization of the membranes can affect viability. An interesting feature of these dyes is that the fluorescence emission intensity of fluorescein depends on the pH, thereby giving additional information about cell metabolism. With a maximal emission at pH 9 and minimal emission at pH 5 changes around the neutral pH can be detected (Johnson and Spence, 2010). Again different protocols for cFDA and cFDA-SE have been reported and generally a short incubation time at higher temperatures is necessary (Table 6.2).

Oxidative stress can also be assessed by flow cytometry. Reactive oxygen and nitrogen species (ROS and RNS) such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO^{\cdot}) are naturally occurring by-products of respiration and oxidation. To protect themselves from those toxic compounds, aerobic organisms use enzymes like superoxide dismutase (SOD) or non-enzymatic anti-

oxidants like glutathione (GSH) to control the level of ROS. Environmental oxidizers, such as UV irradiation or chlorination can increase intracellular ROS levels leading to increased oxidative stress and eventually to cell death. To measure the increase of ROS, dihydroethidium (hydroethidine; HE) can be used. The oxidation of HE results in the formation of ethidium, a fluorescent compound that intercalates DNA (ex./em. 520/610nm) (Munzel et al., 2002). Research has shown that not only $O_2^{\cdot-}$ but also cytochrome c and other reactive oxygen and nitrogen species can oxidize HE (Tarpey et al., 2004). The relative reactivities are $ONOO^{\cdot-} > Fe(II)/H_2O_2$ (i.e. HO^{\cdot}) $> O_2^{\cdot-} > H_2O_2$ (Murrant and Reid, 2001) and show that HE provides an indication of both ROS and RNS production (Gomes et al., 2005). Literature on the use of HE for assessing oxidative stress in bacteria by flow cytometry is scarce and staining protocols used concentrations between 0.07 and 31 μM (Herrera et al., 2002; Arku et al., 2011) and incubation times between 0 and 10 minutes at room temperature (Herrera et al., 2002; Baatout et al., 2005) (Table 6.2).

Although lipid composition is not related to cell viability, it is an interesting parameter to investigate cell physiology. Lipid stains can roughly be divided into two groups: lipid analogues and lipophilic organic molecules. The BODIPY-labeled fatty acid analogues are often used for mammalian cells and microscopy, their use in flow cytometry applications is rather rare (Papadimitriou et al., 2007; Benincasa et al., 2009). An example of the second class is Nile red (NR), which binds selectively to non-polar lipid droplet inside cells (Johnson and Spence, 2010) and can be used to detect the presence of storage lipids (PHA/PHB) in spectrophotometry (Greenspan and Fowler, 1985) and flow cytometry (Ackermann et al., 1995; Gorenflo et al., 1999; Vidal-Mas et al., 2001; Herrera et al., 2002; Tyo et al., 2006). Tyo et al. (2006) showed that ionic strength of the dilution buffer influenced staining efficiency and recommended to use deionized water as dilution buffer instead of physiological saline solution (0.9% NaCl) to improve signal to noise ratio. The authors also mentioned the need for membrane permeabilization for specific bacterial species, which reduced viability. Interestingly, they also showed that the optimal concentration of NR is species dependent potentially because of differences in PHA contents. Protocols vary with concentrations between 3 and 100 μM (Herrera et al., 2002; Tyo et al., 2006) and incubation times ranging from 10 minutes to 30 minutes at room temperature (Gorenflo et al., 1999; Tyo et al., 2006) (Table 6.2).

Similar to the cell-permeant nucleic acid dyes, we first assessed staining kinetics for the different dyes summarized in Table 6.3 (Appendix 2). For most dyes it was found that dye uptake was time-dependent but after fluorescence intensity maximized, it remained stable until the end of the measurement (40 min). In contrast to the other functional dyes tested, HE showed a time-independent uptake in the Gram-positive population. For DiBAC₄(3), the minimal incubation time was 22 minutes regardless of the type of bacteria

6.4 Stain combinations

Combining different stains offers the possibility to simultaneously assess different physiological states of bacteria within a population, thereby improving understanding of bacterial behavior within a specific condition (Nebe-von-Caron et al., 2000; Nielsen et al., 2009; Rezaeinejad and Ivanov, 2011). Several studies used and described double and triple staining protocols in order to determine different functional properties of a bacterial community (Hewitt et al., 1999; Johnson and Spence, 2010). A widely used stain combination is available in the commercialized Live/Dead BacLight kit (Thermo Fisher Scientific, USA), which uses a combination of SYTO 9 and PI to distinguish intact 'live' cells from permeabilized 'dead' cells. This kit has been used in numerous studies (Lawrence et al., 1998; Alonso et al., 2002; Mah et al., 2003; Dalwai et al., 2006; Leys et al., 2009; Vriezen et al., 2012). Table 6.4 gives an overview of genuine double and triple staining protocols and their applications. For these combinations, many multicolor protocols have been optimized in which dyes are separately added to different technical replicates of the same sample. This is different from a genuine multicolor setup in which dyes are added to the sample simultaneously.

In order to combine different dyes, it is important to choose dyes that possess the right spectral properties, to determine the incubation time, incubation conditions and dye concentration for each fluorescent probe separately, and to assess possible interference. When combining stains, one of the most common issues is overspill, which is a consequence of the spectral characteristics of the dyes. To resolve overspill other dye combinations can be made or compensation can be applied. A second type of interference, also related to the spectral characteristics of the dyes, is FRET (fluorescence resonance electron transfer). In this case the emission of one dye (donor) is absorbed by a second dye (acceptor) in close proximity. As a consequence,

the fluorescence intensity of the donor decreases (quenching) and the fluorescence intensity of the acceptor increases (Horváth et al., 2005). Besides FRET, also the matrix can cause fluorescence quenching and decrease in fluorescent intensity. Each dye combination behaves differently within a certain combination of medium matrix, microorganism or instrument and needs to be tuned to determine possible compensations and obtain reproducible results (Hyka et al., 2010; Tracy et al., 2010). To test the feasibility of 'true' triple staining protocols, stain combinations were chosen on the basis of spectral characteristics. A green fluorescent stain (DiBAC₄(3), cFDA and cFDA-SE) was combined with an orange fluorescent stain (HE, NR) and a red fluorescent DNA counterstain (SYTO 60). Several important parameters such as distinguishable populations, total cell concentration, interference between stains and overspill in other channels were evaluated and a gating protocol was established. This has been further illustrated in Figure 6.2.

Four different *C. metallidurans* or *L. brevis* suspensions were made (i.e. a heat-killed, a peroxide-exposed, a stationary phase and a mixed population), which were subsequently stained either with a single stain or all combinations of double and triple stains (Appendix 2). This approach was necessary to determine the appropriate compensation, thresholds and gating. For all triple stain combinations, four populations could be identified with flow cytometry. The threshold and the number of events detected were always affected when stains were combined because of the increased background to signal ratio as a result of spectral overlap and compensation. In addition, compensation was necessary for all green and orange fluorescent dyes, as all caused overspill in the other fluorescent channels. DiBAC₄(3) caused the most spillover and a compensation of more than 100% was necessary making this dye unsuitable in combination with NR and HE. Both cFDA and cFDA-SE showed to be more suitable for triple staining. Combinations with NR required a 25% and 15% compensation in combination with cFDA and cFDA-SE, respectively. This is slightly higher than for HE, which required a 9% and 13% compensation in combination with cFDA and cFDA-SE, respectively. SYTO 60 did not require compensation and allowed to distinguish cells from the background as well as correcting for false positive and false negative events. Because of this, any combination of a functional dye was possible with SYTO 60, resulting in more reliable population counts.

Table 6.3: Overview of the results obtained for time measurements of the different DNA and functional stains.

[illegible]

Table 6.4: Overview of double and triple combination of stains, setup of combinations and subject in which they were used.

stain	setup	subject	reference
Hexidium iodide/cFDA	Double stain	Microbial fermentation	Hewitt and Nebe-Von-Caron (2001)
cFDA/PI/DiBAC ₄ (3)	Dual single DiBAC ₄ (3) cFDA/PI	Bile salt stressed bifidobacteria population	Amor et al. (2002b)
cFDA/PI	Dual	Heating, freezing, low pH, or bile salt exposed cells	Bunthof et al. (1999a)
cFDA/PI	Dual	Gastro-intestinal stresses on probiotic bacteria	Chen et al. (2012a)
Rh123/ DiBAC ₄ (3)/PI/SYTO13/SYTO17	Dual PI/SYTO13 single other stains	Gramicidin, formaldehyde, and surfactants effects on <i>Escherichia coli</i>	Comas and Vives-Rego (1997b)
SYTO9/PI+cFDA/Hoechst3342/resazurine/SYTOX green	Dual	Growth phase-related changes in <i>Bacillus cereus</i>	Cronin and Wilkinson (2008b)
Hexidium iodide/cFDA	Dual	Activated sludge bacteria	Forster et al. (2002a)
CTC/cFDA/DAPI	Dual CTC/DAPI and cFDA/DAPI	Pharmaceutical water supply system bacteria	Kawai et al. (1999)
PI/DIOC ₆	Dual	Starvation response in <i>Bacillus licheniformis</i>	da Silva et al. (2005)

Rh123/DiBAC ₄ (3)/PI	Dual Rh123/PI and DiBAC ₄ (3)/PI	Starvation survival in seawater of <i>Escherichia coli</i> and <i>Salmonella typhimurium</i>	Lopez-Amoros et al. (1995)
Thiazoleorange(TO)/PI/ DiBAC ₄ (3)	Dual Thiazoleorange(TO)/PI and single DiBAC ₄ (3)	Characterization of bacteria in microbial fuel cells	Matos and da Silva (2013)
SYBR green/EB/ DiBAC ₄ (3)/PI	Dual SYBR green/PI triple SYBR green/EB/DiBAC ₄ (3)	<i>Pseudomonas fluorescens</i> DR54 biocontrol	Nielsen et al. (2009)
PI/cFDA/ DiBAC ₄ (3)	Dual PI/cFDA single DiBAC ₄ (3)	Acid stress of <i>Streptococcus macedonicus</i>	Papadimitriou et al. (2007)
CTC/fluorescent-antibody /DAPI	Dual CTC/fluorescent-antibody single DAPI	Enumeration of enterohemorrhagic <i>Escherichia coli</i> O157:H7	Pyle et al. (1995)
cFDA/cFDA-SE/PI/ DiBAC ₄ (3)	Dual cFDA/PI single DiBAC ₄ (3) and cFDA-SE	Fermentation of <i>Lactobacillus delbrueckii</i>	Rault et al. (2008)
cFDA/PI	Dual	Viability of <i>Escherichia coli</i> O157:H7 in river water	Tanaka et al. (2000)
cFDA/PI/CTC	Dual cFDA/PI and single CTC	Natural river water samples	Yamaguchi and Nasu (1997)

Chapter 6

It thus can be concluded that the simultaneous discrimination of certain physiological states is possible when protocols and staining conditions are optimized and appropriate compensation is set. The addition of a red excited nucleic acid dye as counterstain reduces the background to signal ratio and improves the separation between the positive and negative populations.

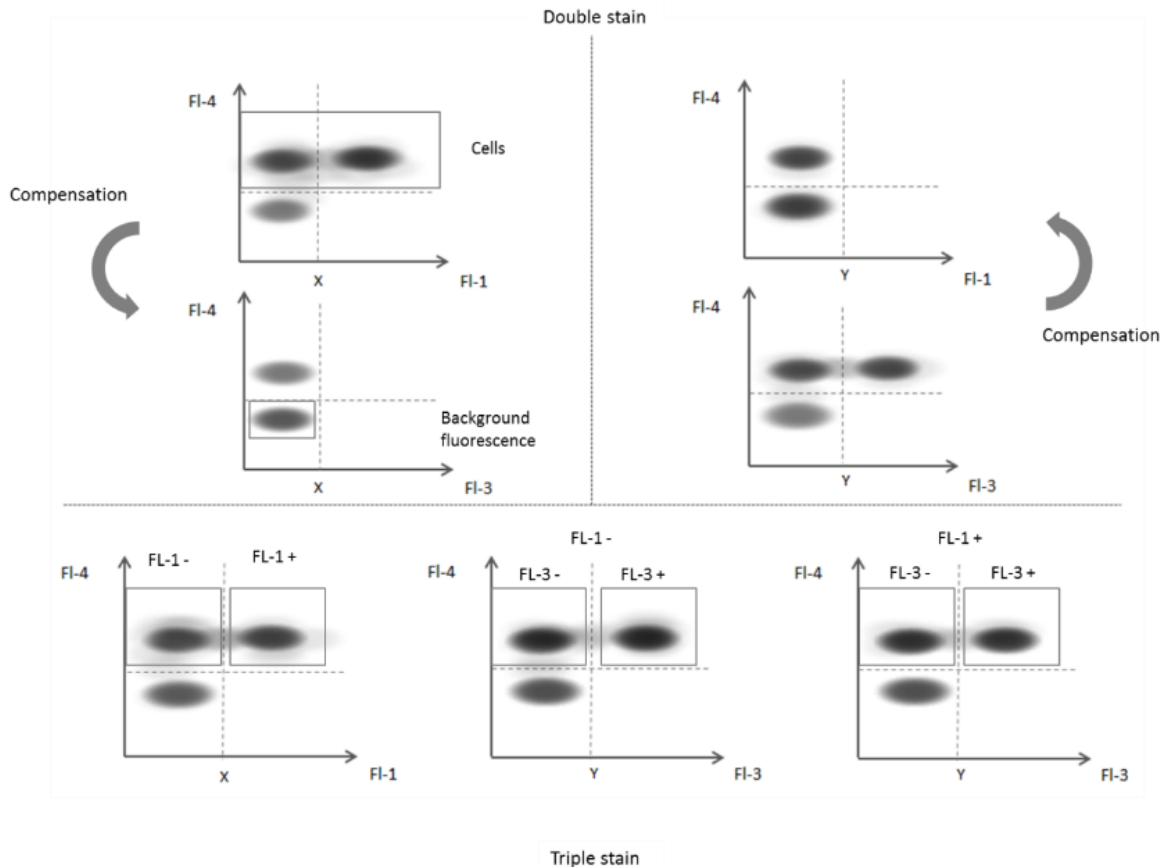


Figure 6.2: Illustration of the gating protocol used to discriminate the 4 populations when using triple stains. The red excitable nucleic acid stain SYTO 60 was used as a counterstain to differentiate cells from background. For the double stains with SYTO 60, first both FL1 positive (upper left) and FL3 positive cells were gated versus FL4 (upper right). Then both positive and negative FL1 and FL3 populations were gated in the opposite channel (e.g. the FL3 positive population was gated in the FL1 channel) to establish the necessary compensation. After the right gates and compensation were established, 4 populations could be distinguished with the triple stains: FL1⁺/FL3⁺, FL1⁺/FL3⁻, FL1⁻/FL3⁺, FL1⁻/FL3⁻.

6.5 Stability

An advantage of flow cytometry is the possibility to analyze samples in a high throughput manner. The use of multiwell autoloaders has become very common and samples are often stained and incubated at the same time. However, this can lead to

discrepancies as samples are not measured simultaneously. In that time frame, biological changes such as aggregation or physiological and chemical changes such as bleaching, dye extrusion or intrusion can occur and significantly alter the results and subsequently affect the reliability and reproducibility (Hyka et al., 2010). Dye stability is therefore an important factor to take into account, particularly when measuring in high throughput screening mode. Only Hammes et al. (2012) and Van Nevel et al. (2013) discussed this issue for the double SYBR green and propidium iodide staining. To assess the stability of the dye, Van Nevel et al. (2013) aliquoted a sample with a known concentration of cells in a multiwell plate after staining with their optimized staining protocol and monitored the mean fluorescence intensity and cell concentration of the detected populations. This way the gradual changes in the results could be detected. They showed that SYBR green I staining is stable for at least 74 minutes, making this stain suitable for multiwell plates. On the other hand, they demonstrated that the combination of SYBR green and propidium iodide is less stable and that, albeit the number of cells in each population remained stable, the fluorescence intensity changed over time. As a consequence, care should be taken when many samples are analyzed in batch and when a fixed gating template (Prest et al., 2013) or flow cytometric fingerprinting (De Roy et al., 2012) is used to analyze the data. The authors also clearly mention that the dye stability is different when other dye concentrations or samples are tested and illustrate the usability of such tests to develop high throughput assays.

We therefore investigated the stability of the previously described triple staining protocols. A synthetic microbial community with different cell populations (e.g. dead, live or oxidized cells) was aliquoted in a 96 well plate, stained and incubated as previously described (Appendix 2). Stability was assessed by comparing the number of cells in the pre-established gates of each sample. As all samples were measured consecutively and for a fixed time (1 minute), the stability in time could be determined. For few triple stains a satisfying degree of stability (10% deviation in the cell counts per gate) for the different populations was found based on the analysis of a 96 well plate. While DiBAC₄(3) is difficult to combine with both NR and HE, only combinations with cFDA and cFDA-SE are considered. As expected, both carboxyfluorescein stains impair the stability of triple stains due to the previously mentioned leakage. Only a maximum stability of 15 minutes was measured making these triple stain combinations

unsuitable for high throughput screening test requiring more than 15 min analysis time. Similar to the results found by Van Nevel et al. (2013), stability can be affected or even improved when other dye combinations or other dye concentrations are used.

6.6 Conclusion

Since the introduction of flow cytometry for research, it has been used to analyze cell populations through high-throughput single cell analysis. Numerous staining protocols have been developed for many applications. However, knowledge about the influence of the different methodological factors on the measurement, and its subsequent interpretation, is still lacking. This is important as they impact the reliability and reproducibility of a staining protocol. Few genuine multicolor protocols have been developed for microbiology and even fewer give an explanation why a certain concentration, incubation time and temperature were chosen or how stable the added fluorochromes were. Furthermore, few results have been published regarding the performance of multicolor protocols in multiwell assays.

We demonstrate how red excitable SYTO dyes can be integrated as a counterstain for multicolor protocols. Moreover, we tested some functional dyes and showed that, like the SYTO dyes, the efficiency differed between the tested organisms, confirming that optimization is necessary for accurate functional measurements. For all tested dyes we showed that their fluorescence intensity remained stable after fluorescence intensity maximized, offering the possibility to combine dyes with different minimal incubation times. However, we found that the combined dyes influence the results and that appropriate controls and compensation are crucial for a correct analysis. DiBAC₄(3) was difficult to combine as it produced a lot of background fluorescence and spillover while cFDA and cFDA-SE were more suitable for combinations. Being excited by a red laser, SYTO 60 did not create spillover and was thus easily combined with other dyes. In general, the addition of a nucleic acid stain improved the results as background fluorescence could be more correctly assessed. Another parameter that influences stain performance is the dye stability during analysis, as the last sample of a batch analysis will not be analyzed at the same time as the first sample and may have undergone changes in biology (e.g. aggregation, sedimentation behavior, physiological changes) as well as in staining chemistry (e.g. bleaching or leakage).

Both literature and our preliminary results clearly show that stability is important to ensure correct results.

6.7 Acknowledgments

This work was supported by the project grant SB-131370 of the Agency for Innovation by Science and Technology (IWT Flanders) (Benjamin Buysschaert), the European Space Agency (ESA-PRODEX), Belgian Science Policy (Belspo) through the E-GEM/BIOROCK project (Bo Byloos) and the Inter-University Attraction Pole (IUAP) “ μ -manager” funded by the Belgian Science Policy (BE, 305 P7/25). The authors want to thank An-Sofie Lerno for the help in the lab and Jeet Varia and Ruben Props for critically reading the manuscript.

Chapter 7

General discussion and future perspectives

General discussion and future perspectives

7.1 General research outcomes and positioning of this research

7.1.1 Positioning of this research

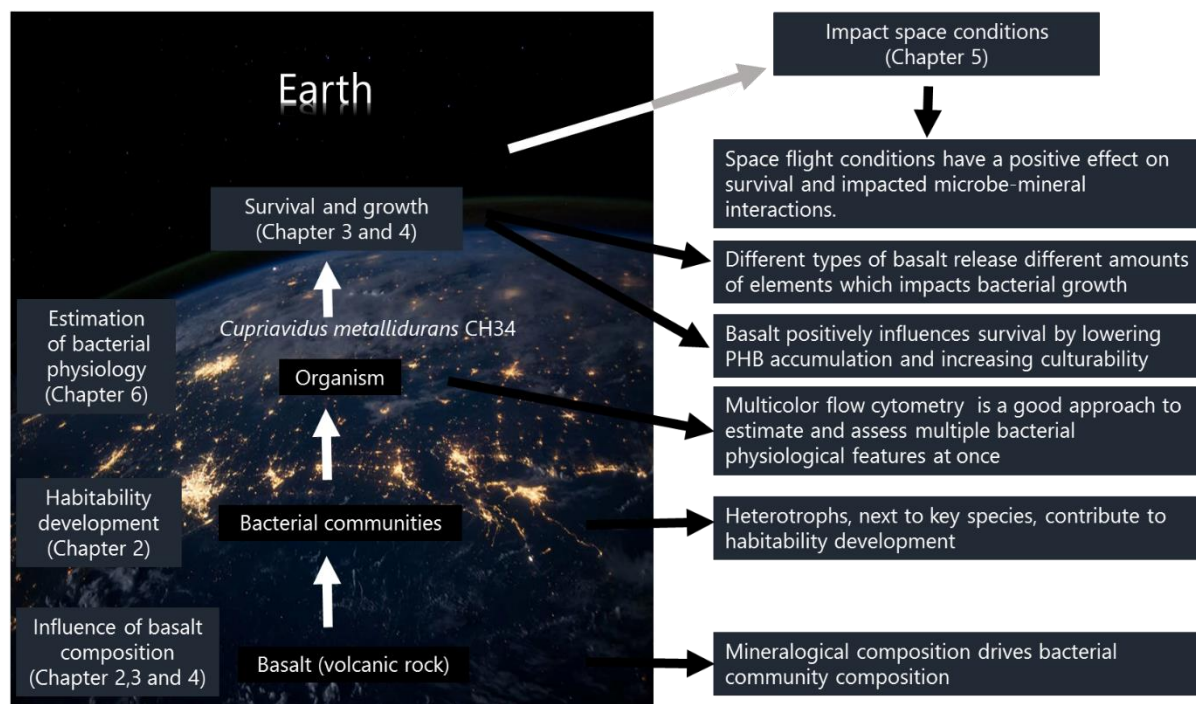
Microbe-mineral interactions, whereby microorganisms interact with minerals for sustaining their survival and growth, impact rock and mineral weathering (Dong, 2010). Microbial colonization of minerals seems to be an ancient strategy (Furnes et al., 2007; Ivarsson et al., 2012) and life on Earth probably originated within a mineral habitat (Ferris, 2005; Uroz et al., 2015). Weathering of crustal rocks is one of the principal processes controlling the geochemical cycling of elements on the Earth's surface. Weathering leads to consumption of atmospheric carbon dioxide and this release or leaching of elements from the rocks are essential for soil formation (Garrels and Mackenzie, 1971; Gadd, 2010). Basalt, a volcanic lunar analog rock, is the most common igneous rock type on the Earth's surface covering in total 60% of the terrestrial surface and ocean floor (Winter, 2014). As these rocks weather more easily compared to other crystalline silicate rocks, their weathering acts as a major atmospheric CO₂ sink (Louvat and Allègre, 1997; Dupré et al., 2003). Microorganisms play a prominent part in the weathering of basalt and glass as shown by laboratory studies (Thorseth et al., 1992; Staudigel et al., 1998; Wu et al., 2007; Staudigel et al., 2008) as well as by *in situ* incubation experiments (Bach and Edwards, 2003).

These interactions can also lead to the formation of microbial biofilm communities on the mineral surface. Biofilms are formed through the preferential colonization of microorganisms on mineral surfaces to increase their survival (Uroz et al., 2009). This colonization is a dynamic process whereby microbial cells attach to solids (such as minerals) and start producing extracellular polymers which provides structure and protection from harsh environments (Characklis and Marshall, 1990; Harrison et al., 2005a). The bacterial community composition that will develop on the rock is impacted by the chemical composition of these rocks through the availability of certain elements

within the microhabitat (Carson et al., 2009; Kelly et al., 2010). Bacteria can also start accumulating intracellular storage polymers such as polyhydroxybutyrates (PHB) and transition into a viable but non-culturable state (VBNC), where bacteria remain viable but lose their culturability on media, to increase their survival (Pedrós-Alió et al., 1990).

Next to their involvement in bioweathering and the geochemical cycle, microbe-mineral interactions have already proved their relevance in industrial applications such as enhancing soil fertility, promoting plant growth, bioremediation and bioremediation of inorganic pollutants and biomining of valuable metals from low grade ores or waste streams (Uboldini et al., 2000a; Mapelli et al., 2012). Microbe-mineral interactions could also have their use in space exploration. At the moment, human presence in space requires full support from Earth. For future more distant space missions, resupply from Earth becomes less evident. Current research is therefore investigating if supplies can be generated from endogenous material on planets and asteroids, so called “*in situ* material”, such as the regolith and rocks (Cockell, 2010). Microorganisms can be exploited in such *in situ* resource utilization (ISRU) processes, for biomining, for life support systems or for enhancement of soil fertility. Since space conditions have been shown to cause many changes in bacterial physiology, including changes in motility and biofilm formation (Brown et al., 2002; Leys et al., 2004; Horneck et al., 2010; Leroy et al., 2010; Kim et al., 2013c), the space environment may also influence microbe-mineral interactions. Therefore, to investigate the impact of space conditions on micro-mineral interactions and to probe for potential applications, the European Space Agency (ESA) has established a Topical Team “Geobiology in Space Exploration” (Cousins and Cockell, 2016). In addition, through the ESA BioRock experiment, which is currently in preparation, scientists will investigate the growth of biofilms and element leaching on a lunar rock analogue (basalt) under near weightlessness and Martian gravity (0.38g) within the ISS.

To increase the knowledge about microbe-based *in situ* resource utilization, and in preparation of the ESA BioRock project, the interaction of the bacterium *Cupriavidus metallidurans* CH34 with basalt, a volcanic moon-analog rock, was investigated. The bacterial presence on basalt and its impact on weathering, as well as the effect of basalt composition on nutrient leaching and bacterial growth, was studied. In addition, the potential impact of space environmental conditions such as microgravity on survival and mineral interaction were determined.



Clostridium, *Chitinophagaceae*, and heterotrophs such as *Ralstonia* appeared to be responsible for the shift of the population from the young to the old flow.

Next, in **chapter three**, we aimed to evaluate the role of basalt composition on nutrient leaching and subsequent support of *C. metallidurans* growth. Growth in medium was affected by magnesium, iron and phosphorus deficiency, resulting in a decreased growth rate and lower final cell density, but was not affected by calcium deficiency. Seven different basalts were examined, each leaching different amounts of calcium, iron, magnesium and phosphorus. In iron-limiting media *C. metallidurans* growth could be restored by all basalt tested, while only two basalts could restore the growth in magnesium-limiting medium. Lack of phosphorus could not be complemented by any of the basalts tested. Except for magnesium, element complementation was not correlated with the composition of the basalt. Thus, different basalt compositions leach different amounts of elements, which are not necessarily correlated with basalt composition, affecting element availability and bacterial growth.

In **chapter four**, during long term storage and starvation conditions, microbe-mineral interactions can become even more relevant for scavenging essential nutrients to support survival. Therefore, the link between nutrient, energy status and element availability of rock, culturability and its impact on biofilm formation and microbe-mineral interactions were studied with *C. metallidurans*. Our results indicated that basalt had a positive effect on survival through the release of elements such as sodium, potassium, magnesium and phosphate, counteracting some of the detrimental effects of starvation. Cells take up these elements while releasing magnesium and calcium in the water leading to a larger culturable fraction and less cells with permeable membranes, while they accumulate less PHB and contained more ATP. Basalt, by releasing elements, lessens the effects of starvation, which leads to higher culturability and activity of these cells. They will therefore not as easily transition into a VBNC state. Cells start forming a biofilm on basalt and PHB could potentially be used for biofilm formation protecting the cells from the harsher planktonic environment.

In **chapter five**, to prepare for a potential application of the ISRU process in space, and in preparation of the ESA BioRock project, further research was done to determine the impact of space flight conditions on the long term storage and microbe-mineral interactions of *C. metallidurans* with basalt. Space flight conditions had a positive effect on survival, which adds to the basalt effect seen from chapter 4, and cells were

more culturable, contained more ATP and more cells were present which were intact and active while fewer cells lost their membrane potential. Copper is released from the basalt only in the presence of cells both in flight and ground, showing that cells have a positive impact on copper release from basalt, while cells were shown to take up phosphate. In space conditions, cells form slightly less biofilm, which could have an impact on microbe-mineral interactions and thus the basalt bioweathering.

In **chapter six**, and in support of our work, we showed that multicolor flow cytometry is a good approach to estimate and assess multiple bacterial physiological features at once and characterize in detail the heterogeneity of a community and bacterial physiology. Numerous staining protocols have been developed for different applications. However, few genuine multicolor protocols have been developed for microbiology and even fewer give an explanation why a certain concentration, incubation time and temperature were chosen or test fluorochrome stability. This is important as it could impact the reliability and reproducibility of a staining protocol. Furthermore, few results have been published regarding the performance of multicolor protocols in multiwell assays. We demonstrated that red-excitable SYTO dyes could be used as counterstain in combination with other functional dyes. The efficiency differed between the tested organisms, indicating that optimization is necessary for accurate functional measurements. Our tests showed that dye combination influenced the results as well as stability and that appropriate controls and compensation are crucial for a correct analysis.

7.2 The impact of basalt age and composition on bacterial communities

Igneous rocks constitute about 95% of the Earth's upper crust where they are subject to weathering. This weathering process regulates the Earth's biosphere, not only now but also in the past during Early Earth formation (Schulz et al., 2013). Basalt has in particular received significant investigation because of its high weathering rates that account for more than 30% of the global carbon dioxide flux, serving as long-term storage of atmospheric CO₂ (Cockell et al., 2009; Summers et al., 2013). However, the initial processes of colonization and subsequent basalt weathering by microbial communities are still poorly understood. New basalt formed as lava deposits after volcanic activity, undergo temperature fluctuations and desiccation, are exposed to higher UV radiation, and lack readily available nutrients imposing highly selective

conditions for colonizing bacterial communities (Staudigel and Hart, 1983; Carson et al., 2009; Kelly et al., 2010; Kelly et al., 2011; Fujimura et al., 2012; Olsson-Francis et al., 2015). Studying these extreme environments and its habitability could be beneficial for understanding these underlying weathering and ecological development processes. Relatively few data are however available on the diversity and richness of the communities successively colonizing chronological terrestrial basalt flows on Earth. Our study showed that the basalt environment is first colonized by key species belonging to the *Acidobacteria*, *Actinobacteria* and *Proteobacteria* phyla (Chapter 2). These species indeed have been associated with the core basalt communities (Gomez-Alvarez et al., 2007; Kelly et al., 2010; Olsson-Francis et al., 2015). *Actinobacteria* especially are an important phylum as members have been shown to resist desiccation and possess oligotrophic growth capabilities (Lechevalier and Lechevalier, 1967; Gomez-Alvarez et al., 2007; Cockell et al., 2013; Summers et al., 2013). In addition, their autotrophic and chemolithotrophic metabolism allows them to fix inorganic C in the basalt environment while impacting P and S release from the basalt, enhancing basalt weathering (Gomez-Alvarez et al., 2007). Both *Arthrobacter* and *Streptomyces* species, which belong to the *Actinobacteria*, also found in our study on the young flows, have indeed been shown to be key species within this environment and were shown to induce rock weathering in different laboratory setups (Frey et al., 2010; Cockell et al., 2013). Older lava deposits contained more heterotrophic and N₂-fixating bacteria such as *Planctomycetaceae*, *Rhizobiales*, *Rhodospirillales* and *Sphingomonadales* (Chapter 2). These groups have already been associated with basalt communities in other volcanic settings (Einen et al., 2006; Henri et al., 2015; Kim et al., 2015; Paul et al., 2015). In our study, the older the lava deposits, the more *Planctomycetaceae*, *Rhizobiales*, *Sphingobacteriales* and *Acetobacteraceae*, *Clostridium*, *Chitinophagaceae*, and heterotrophs such as *Ralstonia*, were dominating the microbial community (Chapter 2). Thus, it seems that, over time, this environment is gradually colonized by heterotrophic N₂-fixating soil and rhizosphere bacteria, which contribute to further weathering of the rock, and finally provide the opportunity for lichen and plant development. It has already been shown that heterotrophic N₂-fixers interact with lichen fungi and that this mutualistic process contributes to enhanced mineral weathering (Seneviratne and Indrasena, 2006). The gradual increase in abundance of heterotrophs with the age of the lava deposit, as observed in our study, indicates that these heterotrophs rely, for the availability of carbon, on the initial key litho-autotrophic

species (primary producers) to make it available in organic form (Cockell et al., 2009). Heterotrophs then further rely on different metabolic capabilities and survival strategies to conquer and thrive in this environment (Walsh and Clarke, 1982; Cockell et al., 2009). These capabilities promotes further weathering and have been shown to enhance obsidian volcanic glass weathering (Cockell et al., 2009; Herrera et al., 2009). It has also been shown by Pokrovsky et al. (2009) that the heterotroph *Pseudomonas aureofaciens* can impact basalt wollastonite mineral weathering through pH alternation. *Burkholderia* species have been shown to produce oxalate as well as EPS which could contribute to rock weathering (Olsson-Francis et al., 2015). Such combinations of primary chemoautotrophs and secondary heterotrophs will also be essential for biological ISRU, for example to extract useful nutrients from the regolith on Mars, and to enhance soil formation for food production (Olsson-Francis and Cockell, 2010a; Verseux et al., 2016a) *Pseudomonas* sp. HerB as a chemolithotrophic iron oxidizer, isolated from a lava tube in the Oregon Cascades, USA was proposed by Popa et al. (2012) to be able to live within a near-surface, icy, volcanic environment, analog to the environment found in the past on Mars, and which could grow on olivine as sole energy source. However, differences in the bacterial community composition have been seen for different volcanic settings, likely because of differences in basalt formation and mineralogy. For example, Hawaiian deposits were dominated by *Cyanobacteria*, *Acidobacteria* and *Alphaproteobacteria* which are different from the Icelandic sessions mentioned earlier (Kelly et al., 2010; Kelly et al., 2014). Further research is necessary to understand the relation between mineralogy, element composition, ecological development and the prevailing bacterial communities.

Differences in rock mineralogy will also affect weathering rates, impact the availability of certain elements within the microhabitat and temperature (through rock albedo) (Wolff-Boenisch et al., 2004; Hall et al., 2005; Carson et al., 2009; Kelly et al., 2011). Icelandic bacterial communities were found to vary on volcanic rocks of different mineralogic composition (Kelly et al., 2010) and within our study, composition analysis of the different basalt formations on the subsequent flows showed a clear variation in FeO and Fe₂O₃ oxides content. These differences could be positively correlated to the variations in the bacterial communities present within these lava deposits. Within terrestrial basalts, iron, as part of the olivine, pyroxene and in glass minerals is present as Fe²⁺ (Edwards et al., 2008). These iron minerals are indeed an important source of

energy (Edwards et al., 2004) as they can serve as an electron donor or acceptor (Mitchell et al., 2013). The reduced iron (FeO) could also become hydrated which leads to hydrogen production and could serve as electron donor for the heterotrophic iron oxidizers as well as methanogens and sulfate reducers (McGrail et al., 2006; Mason et al., 2009) e.g. *Methylobacterium* and *Desulfovibrio*, respectively, found in our study within the more weathered basalt formations (Chapter 2). Herrera et al. (2008) also showed that bacterial communities colonizing Icelandic obsidian volcanic glass were localized around iron and pyroxene phenocrysts showing that within the terrestrial basalt environment this could be an important element for colonization. This has also been shown for the bacterial communities present within ocean basalt floors where iron oxidizing bacteria are key species within this environment (Edwards et al., 2005; Cockell et al., 2011). However, the role of this shift in iron oxide content within each of the flows, could be one of the factors contributing to the shift between the different flows. Other factors, such as the spatial heterogeneity, the age of the deposit as well as the associated carbon content and exogenous rain precipitations and wind can influence this as well. In addition, factors such as porosity, pH and surface characteristics of these lava flows have also been shown to influence bacterial community composition (Barker et al., 1998; Bennett et al., 2001).

7.3 Basalt as source of nutrients for bacterial growth

In this study, we used the chemolithoautotrophic and facultative heterotrophic bacterium *Cupriavidus metallidurans* CH34 to further study microbe-basalt interactions more in detail. It was already shown that *Cupriavidus* species are part of the early colonizers on newly formed volcanic substrates such as lava in the Philippines (Sato et al., 2006; Kelly et al., 2010). This was also confirmed in our study, where they were found as members of the microbial community of the medium and old flows that we studied (Chapter 2). Type strain *Cupriavidus metallidurans* CH34 was also already tested in the lab with basalt by Olsson-Francis et al. (2010b) as well as by Bryce et al. (2016). In addition, its full genome has been annotated (Janssen et al., 2010) and it has been shown to contain metal resistance proteins as well as possess a broad metabolic diversity (Mijnendonckx et al., 2011; Van Houdt et al., 2012; Chalia et al., 2016; Nies, 2016). Therefore, *Cupriavidus metallidurans* CH34 was selected as valid model organism in our geomicrobiological study.

The influence of rock composition on the microbial development and bioweathering is still poorly understood. Our study could show that different basalts with different compositions, leach different amount of elements (Chapter 3). All basalts leached sufficient iron to fully support growth of *C. metallidurans* in iron-depleted medium, but only two basalts tested could restore the growth in magnesium-limiting medium (Chapter 3). The fact that basalt releases iron and can provide essential elements needed for *C. metallidurans* growth was also seen by Bryce et al. (2016). Strain CH34 typically produces siderophores when grown in iron-limited medium, but not when basalt was added. With basalt it upregulated the expression of the ABC transporter genes which are involved in Fe^{2+} sequestering, enabling *C. metallidurans* of acquiring iron released from basalt (Olsson-Francis et al., 2010). That basalt can have a positive effect on bacterial growth has also been reported for other bacteria. Batch experiments with *Pseudomonas stutzeri* VS-10, isolated from seafloor volcanic rocks, indicated elevated growth of this isolate in the presence of basalt. The authors concluded that most likely the physical contact, or at least proximity to basaltic glass, increased growth by increasing its iron oxidation activity (Sudek et al., 2017).

Our study showed subtle differences on growth, as growth rates were slightly affected in the presence of the different basalts tested (Chapter 3). Using a similar setup, but with a different basalt composition, Bryce et al. (2016) observed an initial pH increase and reduction in total dissolved phosphate and phosphorus concentrations in the abiotic controls due to phosphate sorption onto the rock via ligand-proton exchange and formation of calciumphosphates (Frossard et al., 1995; Stockmann et al., 2011). Both the pH increase and phosphorus sequestration diminished growth of *C. metallidurans* in their setup. Although *C. metallidurans* counteracted this phosphate limitation by increasing the expression of phosphate importers and utilizing intracellular phosphate reserves, growth could not be fully restored (Bryce et al., 2016).

These observations clearly indicate that basalt, depending on its composition, can impact bacterial growth through element release. *C. metallidurans* will possibly counteract this element limitation directly by upregulating transporters and influencing saturations states of elements. In conclusion, basalt composition should thus be taken into account as a parameter when considering further applications and its use as a nutrient source.

7.4 Basalt as source of nutrients for survival and substrate for biofilm formation

During long-term storage and starvation conditions, microbe-mineral interactions can become even more important as released elements serve as nutrients, retaining of their energy status in order to support survival (Dong, 2010). Bacteria undergoing nutrient starvation conditions and other stresses become smaller, undergo changes in their cell membrane composition, show lower activity and become more resistant to adverse environmental conditions (Malmcrona-Friberg et al., 1986; Trevors, 2011; Su et al., 2015). Bacteria undergoing these conditions can also transition into a 'viable but non-culturable' state (VBNC) where bacteria remain viable but lose their culturability on growth media. This 'VBNC' state is shown to be induced by a variety of stresses such as pH, temperature and starvation and has been identified in 85 different species (Ayrapetyan and Oliver, 2016). Recovery of these non-culturable bacteria has been difficult to prove, produce and reproduce (Lopez-Amoros et al., 1995; Gasol and Del Giorgio, 2000; Amor et al., 2002a; Falcioni et al., 2008; Hammes et al., 2011; Boi et al., 2015). Some of these bacteria resuscitate when the inducing stress is removed, while the involvement of other growing bacteria and quorum sensing molecules can be important for others (Oliver, 2016). The physiological basis for these processes is not yet fully elucidated, (Kell et al., 1998; Breeuwer and Abee, 2000b; Epstein, 2013; Oliver, 2016). We showed in this study that multicolor flow cytometry could be a good technique to estimate and assess multiple physiological features at once and be used to characterize in detail bacterial physiology. This technique is used throughout the whole work to assess the effect of the different parameters on *Cupriavidus metallidurans* CH34 physiology and determine its viability in water with and without basalt rock. However, our work also showed that appropriate controls and compensation are crucial for a correct analysis and that no single technique or stain is suited for viability measurements of all species and under all conditions (Davey, 2011). The combination of different techniques is probably necessary in order to fully characterize physiological differences and heterogeneity.

In our study, long-term storage of CH34 cells in mineral water had, as expected, a significantly negative effect on viability, energy status and physiology, leading to cells transitioning into a VBNC state and a drop in culturability (Chapter 4 and 5). The cells had an impact on the concentration of elements in water, due to the fact that more cells

are permeabilized, and more magnesium, iron and phosphate were present in the water (Chapter 4 and 5). The drop in culturability in water was significant after 7 weeks and was followed by a 25-fold decrease in readily available intracellular energy levels (ATP), and a four-fold increase in energy stock levels (the lipid PHB) over the 12 week experiment, thus affecting the energy status of the cells. The effect of starvation on culturability and ATP levels has already been shown in other experiments in similar oligotrophic conditions (Kell et al., 1998; Oliver, 2005). The composition of the water could influence this as well, as different compositions of river water, impacted the culturability level of *Ralstonia eutropha* (López et al., 1995). It was shown that *C. metallidurans* and closely related *Ralstonia pickettii* isolates can survive in mineral water, even in the presence of antimicrobial silver, for almost two years, but also those cells showed a decline in culturability from 10^9 CFU/ml to 10^6 CFU/ml (Mijnendonckx et al., 2013). *Ralstonia solanacearum* also showed this transition into the VBNC state in river water (Álvarez et al., 2008) or when exposed to high concentrations of copper, low temperature or in soil (Grey and Steck, 2001; van Overbeek et al., 2004; Caruso et al., 2005).

Microbe-mineral interactions can enhance survival, during long term storage and starvation conditions, as minerals can serve as element and/or energy donors (Dong, 2010). Environmental fluctuations as well as the basalt composition have an impact on recovery of these starved cells. Although culturability was lower when cells were incubated with basalt SCK4 and exposed to temperature fluctuations during storage (chapter 5), both basalts tested could counteract the loss in culturability (chapter 4 and 5). In the presence of basalt SCK6 cells maintained a higher operational energy level (ATP) and stored less energy (in PHB), which led to higher culturability (chapter 4). This recovery is due to the fact that these basalts release different amounts of elements such as sodium, potassium, phosphate and iron in the water, also observed in the growth experiment (chapter 3). All basalts tested showed an initial decline of calcium due to complexation on basalt (Stockmann et al., 2011). Cells in water in the presence of SCK6, showed that they could counteract the complexation of calcium and release part of it in the water as well as release magnesium while cells take up the potassium, sodium and phosphate (Chapter 4). For SCK4, no significant differences were seen with cells in the presence of basalt, except for copper and phosphate, and cells increased the release of copper while taking up phosphate (Chapter 6). This difference

in element release, maybe due to differences in phosphate release of the different basalts, leads thus to differences in recovery of starved cells. However, cell culturability still declines in the presence of basalt, indicating that the presence of basalt cannot fully prevent entry into the VBNC state and the precise trigger(s) for entry still remain to be elucidated.

Accumulation of intracellular storage polymers has been shown to be one of the strategies deployed by bacteria to increase their survival as it regulates the amount of 'stored' energy (Pedrós-Alió et al., 1990). One of the most predominant forms of these intracellular storage polymers for *C. metallidurans*, are poly-3-hydroxybutyrates (PHB), which are highly reduced carbon and energy storage compounds, and their synthesis is coupled to carbon and nitrogen metabolism. These are also involved in cellular redox balance as these polymers also act as a sink for reducing equivalents (Raberg et al., 2014;López et al., 2015). Previous studies showed that bacterial cells accumulate PHB in nutrient poor environments such as in mineral water (Kadouri et al., 2005). PHB accumulation could result from metabolic redirecting of protein or lipid cellular fractions as seen in closely related *Ralstonia eutropha* H16 (Brigham et al., 2010;Sharma et al., 2016). Factors such as exposure to stress conditions (Povolo and Casella, 2000;Rojas et al., 2011) phosphate limitation (Shang et al., 2003;Budde et al., 2011) and elevated C/N ratios (Park et al., 2011) can trigger PHB accumulation as well. Our results reflect that basalt could compensate for loss in the culturability while cells accumulate less PHB, maybe due to phosphate release (Chapter 4). Producing and degrading PHB improves fitness of rhizospheric bacteria as it stabilizes the intracellular redox conditions and compensates TCA cycle inhibition in low oxygen conditions (Anderson and Dawes, 1990;Dunn, 1998). This PHB utilization leads to a more balanced growth rate and can give a competitive advantage in surviving these environments over organisms without this storage capability (Beun et al., 2002). Impaired PHB accumulation in *Ralstonia eutropha*, by construction of PHB-negative cells, leads to loss of culturability compared to the parental strain (López et al., 1995;Nowroth et al., 2016). This effect on culturability is, however, not observed for PHB-negative cells of CH34 (Chapter 5). Basalt could partly recover culturability, reducing the impact of starvation, but not to the level seen in the parental strain. The exact role of PHB within recovery of the VBNC and the trigger for entry into this state remains to be clarified.

The transition from a planktonic mode of survival to a biofilm mode was shown to be evolutionary beneficial into colonizing these more nutrient rich surfaces and establishing more favorable biofilm structures (Jefferson, 2004). Biofilm formation, already started from 4 weeks on basalt which was shown by the presence of polysaccharides, eDNA and protein complexes accumulating on the basalt surface, as seen with fluorescent microscopy (Chapter 5). The trigger for biofilm formation may thus differ from that seen for entry in the VBNC state by the planktonic cells, as this transition happens earlier during storage. This may be due to the fact that basalt can release additional elements through increased EPS solubilisation and increased rock weathering or by impacting the metal-proton exchange at the surface (Bennett et al., 2001; Gislason and Oelkers, 2003; Gorbushina, 2007). Basalt could also act as a source of energy by releasing H₂, as seen in our basalt community study (Chapter 2). However this H₂ release only occurs at pH's equal or lower than pH 6, conditions which are present within Icelandic soils (Arnalds, 2015), possibly within our community study (Chapter 2), but not within our survival setup as pH increases to pH 8 (Anderson et al., 1998).

During long-term storage, biofilm cells differ from planktonic ones as culturability was 1 log lower than that of planktonic cells. Energy levels (both ATP and PHB levels) were 25 and 2 times lower, respectively, than that observed for the planktonic fraction. Both basalt composition as well environmental conditions can impact this and cells incubated with SCK4 and exposed to temperature fluctuations, show a higher decline in culturability while more cells are present within the biofilm (Chapter 5) compared to cells incubated with SCK6 (Chapter 4). It has already been shown that biofilm forming capacity of microorganisms depends upon the surface attributes of the substratum and can vary according to the prevailing environmental and nutritional conditions (Stepanović et al., 2004). However, cells incubated with both kinds of basalt, are less culturable compared to their planktonic fraction, even while the total cell amount remains high. It has already been shown that cells lose their culturability, and transition into a VBNC state when present in biofilm and this is lower compared to the planktonic fraction (Prakash et al., 2003; Manuel et al., 2007). However, less biofilm cells were permeabilized, while ATP content remain constant, for both types tested, which may indicate that the biofilm mode of these cells protects them from the harsher survival conditions in the planktonic fraction (Marshall, 1988; Prakash et al., 2003). This is due

to the fact that biofilm through its gel-like matrix can sequester nutrients, buffer nutrient fluctuations, and establish a more favorable environment (Jefferson, 2004). There may also be a link between biofilm formation and PHB accumulation and Tribelli and López (2011) already showed that PHB accumulation is necessary for biofilm formation. It has also been seen that *Sinorhizobium meliloti* PHB negative cells can no longer produce the extracellular polysaccharide (EPS) succinoglycan and they hypothesized that their production is linked within the same regulatory circuit (Aneja et al., 2004). Within our study, the total cell amount of the biofilm cells declined by one log for the PHB-negative mutant compared to the parental strain, showing that without PHB, less cells transition into the biofilm mode of survival. However, in none of the conditions a decline in PHB content was observed for the parental strain (chapter 4 and 5). Our study indicated that biofilm formation on rocks, such as basalt, is part of the survival strategy, protecting bacteria from the harsher conditions and that PHB metabolism could potentially play a role in biofilm formation. However, the role of PHB and the trigger for biofilm formation on basalt needs to be investigated further.

Space conditions have shown to induce changes in phenotypic microbial characteristics such as growth, morphology, genetic transfer, stress susceptibility and virulence in a number of organisms (Brown et al., 2002; Leys et al., 2004; Horneck et al., 2010; Tirumalai et al., 2017). Changes were also seen in biofilm formation and motility and these may also alter microbe-mineral interactions (Benoit and Klaus, 2007; Kim et al., 2013b). The reduction of gravity eliminates mass driven convection and sedimentation resulting in diffusion as the predominant way in which the microenvironment of the bacterial cells can be changed during space flight (Klaus et al., 1997; János et al., 2002). During our space flight experiment, no significant differences were seen in element release with basalt in water without cells, showing that space flight conditions do not impact the abiotic release of these elements from basalt. When cells were stored in water in space, cells remained relatively intact and leaked less magnesium and iron in the water, irrespective of the presence of basalt. In the presence of basalt, cells release copper, as seen for the ground experiment as well, and take up more phosphate (Chapter 5). Flight conditions, even without basalt, showed a positive effect on cell survival, maybe due to the fact that reduced microgravity, and thus lack of convection and sedimentation, leads to less competition for these cells to regulate their environment, as fluid dynamics limit extra cellular

transport transfer and more elements are available to sustain their survival (Leys et al., 2004). Basalt adds to these effects, again by releasing phosphate and subsequent uptake by the cells, leading to more culturable cells, a higher amount of total, intact and active cells and fewer cells that lost their membrane potential (Chapter 5). Space flight conditions made that biofilm formation was less developed and the biofilm was differently structured. Cells which were present in the biofilm during space flight showed no significant changes in physiology or energy content compared to cells on ground, indicating that space flight does not impact cells once they transition into the biofilm state (Chapter 5). These results, however, remain preliminary and further experiments, such as BioRock, are necessary to further elucidate the effect of space flight conditions on microbe-mineral interactions. For further applications, not only released elements such as calcium, magnesium or copper but also full bacterial biomass could be used for feeding other processes and experiments done with cyanobacterial biomass showed that this biomass could be used a substrate for ethanol producing yeast and further fuel production (Aikawa et al. 2013; Möllers et al. 2014). Also PHB and other biopolymers, which accumulated in cells, could be used as precursor for 3D printing of habitats (Menezes et al., 2015a).

This study could thus show that *C. metallidurans* can survive long term starvation and storage in water, that basalt has a positive impact on viability and culturability, and that PHB metabolism may impact biofilm formation. The kind of basalt influences element release, and *C. metallidurans* cells can stimulate calcium, iron, magnesium and copper release. In addition, space flight conditions positively influences survival and culturability. It is clear that the interaction with basalt and biofilm formation are different for non-actively-growing stationary-phase cells than for actively growing cells, as in growth conditions the amount of elements released is higher and no biofilm is formed, while during long-term starvation experiments the amount of leached elements is lower, potentially lessening the effect of starvation and the transition into a VBNC state, and biofilm formation was seen in order for the cells to be protected from the harsh oligotrophic environment.

7.5 Future perspectives and applications

Geomicrobiology has expanded our knowledge of the microbial presence in the geosphere and its connection with the physical and chemical characteristics of an

environment. It has allowed the development of diverse applications such as biomining of valuable metals from low grade ores or waste streams and enhancement of soil fertility (Dong, 2010). This has also led to a better understanding of the role of microorganisms during Early Earth habitability development (Newman and Banfield, 2002). However, microbe-mineral interactions are still not fully characterized and subject of further investigation.

Based on our results, it is clear that *Cupriavidus metallidurans* CH34 accelerates element release directly, by influencing the saturation states of different elements. However, depending on the basalt composition, uptake is different, and *C. metallidurans* could take up iron and magnesium to restore growth. Within the survival setup, release of magnesium, calcium as well as copper is seen. The use of *C. metallidurans* as biomining organism thus seems limited, except for maybe copper, and more research could be targeted at intracellular accumulation of specific elements. In addition, they are able to produce different amino acids which could induce additional indirect leaching (Janssen et al., 2010). They have also been used within bioremediation studies of mercury, zinc, lead and cadmium (Diels et al., 2009) and as basalt can contain high concentrations of these elements, it could be interesting to further study this for bioremediation with basalt. In addition, also the use of flow cells to induce biofilm formation and increase the release of specific elements could be an interesting approach. Thus, *C. metallidurans* CH34 will accelerate element release, in these conditions, by putatively counteracting element limitation directly by acquisition of major elements inducing weathering of basalt rock. The presence of *C. metallidurans* next to the key species (chapter 2) could indicate that they also have a role in weathering and are able to increase the habitability of these basalt environments. In this respect, strains isolated from basalt (chapter 2) could be further investigated for biofilm formation as well as metabolic activities and the potential use and role for *C. metallidurans* within terraforming applications should be investigated.

More generally, as relatively few data are available on the diversity and richness of the communities inhabiting successive and chronological terrestrial basaltic flows and more information on the different bacterial compositions within different environments is necessary as differences in the bacterial community composition have been seen for different locations (Kelly et al., 2010; Kelly et al., 2014). Molecular biology techniques can be used to assess microbial communities in different locations and

settings, both in terms of their diversity and population sizes. Bacteria clearly undergo different physiological changes in the presence of rocks, such as changes in culturability, ATP and PHB levels. Most of the molecular biological data to explain these changes are still lacking and for many microbe-mineral interactions these biological changes remain unclear and should be studied (Newman and Banfield, 2002; Edwards and Bazylinski, 2008). Genomic and metagenomics sequencing of relevant microbial species and communities can give further insights into the metabolic pathways and activities involved in mineral weathering (Hutchens, 2009). As shown in Chapter 2, basalt composition can also influence bacterial community compositions, thus different basalt surfaces should be investigated and the effect on bacterial community composition studied. The use of atomic force microscopy (AFM) could hereby provide information about the position of the different minerals at the surface, as it allows viewing and manipulating microbial surfaces in their native environments, which is not possible with conventional techniques (such as SEM coupled to energy dispersive X-ray spectroscopy), leading to qualitative and quantitative investigations of native mineral–biofilm interactions (Lower et al., 2000; Dufrêne, 2002). Not only community composition but also community heterogeneity and function need to be further studied, as these are necessary to get a full understanding of microbial behavior and enhance its applications (Hammes et al., 2011). Both micro-array and proteomics have been used to study the molecular cellular changes in detail and this has led to insights of bacterial physiology in contact with the basalt surface (Olsson-Francis et al., 2010b; Bryce et al., 2016). Also other omics technologies could help in better understanding of the response of both a bacterial community and single organisms, by integrating transcriptomics (RNA seq) and metabolomics with metagenomics profiling (Franzosa et al., 2015). We could already show that multicolor flow cytometry is a suitable technique to monitor cell counts, cell viability and cell physiology, but one approach will not be sufficient. More information can be obtained by using for example stable isotope probing (SIP), as it can identify organisms carrying out specific metabolic processes, within different and changing environments (Radajewski et al., 2000). The combined use of SIP with qPCR and taxonomic distribution could show a link between taxonomic diversity and its function within a microbial community (Zhi et al., 2014). Raman spectroscopy is another useful technique for geomicrobiology as it can not only be used to characterize bacterial community composition and its heterogeneity but can

also be used for characterizing the surface and its difference in mineral composition (Huang et al., 2007).

The gained knowledge could also be used for future space applications, to assess habitability development on extraterrestrial planets (Fridlund and Kaltenegger, 2008). It could lead, in the long term, to terraforming and the recovery of nutrients for future extraterrestrial outposts. For example, by combining organisms and planetary basalt rock for transforming the planetary regolith, the planet could be transformed into a more habitable one (Cockell, 2010). Present day conditions on Mars and on the Moon, with high radiation, extreme temperature fluctuations and thin atmosphere make it unsuitable for life (Aubrey et al., 2006; Dartnell et al., 2007; Popa et al., 2012). For ISRU applications, cyanobacteria, have received much attention and have already been studied in astrobiological relevant conditions (Verseux et al., 2016a). Also heterotrophs have been studied, especially *Burkholderia*. However, a more diverse spectrum of bacteria should be tested in these conditions. They are probably also essential, shown by our study, next to the primary producers to enhance soil formation to allow plant cultivation (Olsson-Francis and Cockell, 2010a; Verseux et al., 2016a). Such heterotrophs and chemoautotrophs can also be useful on Mars, to extract special and/or useful nutrients, in combination with the primary producers. Synthetic biology as well as directed evolution could offer another way of engineering organisms for specific purposes and to construct more suited communities (Cockell, 2011; Menezes et al., 2015b; Verseux et al., 2016b). Superweathering microorganisms (SWeMs) that are capable of carrying out rapid mineral dissolution could be created through synthetic construction (Cockell, 2011; Menezes et al., 2015a). Also synthetic communities could be constructed. Mautner (2002) combined *Arthrobacter* and *Nocardia* with the heterotroph *Pseudomonas* and showed that meteorite substrates could support their growth. They also saw that the heterotrophic organisms could directly use the carbon present in the meteoritic extracts, but no additional advantage was seen by the combination of the organisms.

In a final stage, bacterial communities could be used to support the growth of plants in future planetary outposts. Microbe-mineral interactions increase water-holding properties, organic carbon and nitrogen contents, and availability of nutrients necessary for plant development (Verseux et al., 2016b). However, a drawback of using plants within these future outposts, would be that they are less efficient than i.e.

cyanobacteria regarding surface, CO₂ and mineral use, are much more sensitive to environmental conditions, require more handling, are more difficult to engineer, take more time to regrow in case of accidental loss and contain inedible and hard-to-recycle parts (Hendrickx and Mergeay, 2007).

Microorganisms could also be used to mine specific elements from planetary material as already shown for *Acidithiobacillus ferrooxidans*, an acidophilic biomining organism that could oxidise iron present within carbonaceous meteorites, as sole source of energy (Rawlings, 2005; Gronstal et al., 2009). Asteroids also contain other interesting elements such as platinum group elements (including palladium and osmium) and valuable metals (copper, nickel and gold) that could be interesting to use in catalysis and chemical syntheses (Busch, 2004). Here, again synthetic biology could help to perform better by increasing the secretion of elemental sequestration molecules and enhance leaching of elements (Cockell, 2010; Verseux et al., 2016a). In addition, other characteristics could be adjusted so they are more tolerable to extremes in pH, temperature, desiccation, radiation, low pressures, as well as to counteract and remain active after fluctuations in these conditions, the ability to store them and handle them without continuous culturing and minimal growth activation.

The knowledge about the response of bacterial communities as well as single organisms, to astrobiology relevant extremes and the impact of storage and survival of microbes under these stresses is also limited. While space travel technologies have made great steps forward in the last half century, current life support technologies are not sufficiently developed for sustaining manned exploratory missions to Mars (Horneck et al., 2010). Recommendations, for future research paths and application of the ISRU process, have been made by the ESA topical team “Geobiology in Space Exploration, and were prioritized as following: (1) Development of new terrestrial space-analogue environments to study the impact of space conditions on microbe-mineral interactions; (2) Better characterization of the enzymatic and biochemical mineral interaction to space conditions; (3) More and longer space-based microbiology experiments, including precursor flight tests; (4) Integration of fieldsite biogeography with laboratory- and field-based research; (5) Miniaturization of existing analytical methods, such as DNA sequencing technology to better characterize interactions not only on ground but also during flight (Cousins and Cockell, 2016). Thus not only the choice of organisms but also the facilities and opportunities to test this should thus

further be developed. Long-term space experiments will be useful for understanding the long-term viability and practicality of utilizing microorganisms in space exploration (Cousins and Cockell, 2016). The lack of experimentation to date is largely due to the inherent complexity of space-based studies. Experiments in the near-term should explore a compromise between abiotic chemical methods of modifying rocks for practical uses, and the use of active microorganisms. It is clear that well-integrated ground-based experiments can significantly support these experiments conducted on space platforms. Planetary simulation facilities should allow studying mineral-microbe interactions under simulated extraterrestrial extremes such as simulated Martian surface, subsurface and radiation environments (Olsson-Francis and Cockell, 2010a). For biomining, reactors could be built and tested within these stimulated environments, to provide information about the reliability of the instruments, the amount of products formed during these processes and product stability. In addition to the technical feasibility, further steps need to be taken in terms of planetary protection, to establish a legal context as well as resolve political issues in terms of the use of ISRU and its application for resource generation (Crawford, 2015; Carpenter et al., 2016; Rettberg et al., 2016). In conclusion, advances have been made to develop possible future applications of bio-based ISRU, terraforming and biomining. However, further advancement will rely on future choices, made in planetary protection, availability of suited research platforms and possible research targets needed for a persistent human presence in space. For ISRU, bacterial communities and single organisms should be selected that are able to grow in extreme conditions, are suitable for genetical engineering, can perform specific well-studied tasks and possess limiting nutritional properties, thereby targeting a balance between an optimal functioning metabolism, resistance against extremes and process yield.

References

References

- Achbergerová, L., and Nahálka, J. (2011). Polyphosphate-an ancient energy source and active metabolic regulator. *Microbial cell factories* 10, 63.
- Ackermann, J.U., Muller, S., Losche, A., Bley, T., and Babel, W. (1995). *Methylobacterium Rhodesianum* Cells Tend to Double the DNA Content under Growth Limitations and Accumulate Phb. *J Biotechnol* 39, 9-20.
- Adams, J., Bhattacharya, M., Lin, Z.-W., Pendleton, G., and Watts, J. (2007). The ionizing radiation environment on the moon. *Advances in Space Research* 40, 338-341.
- Aiuppa, A., Allard, P., D'alessandro, W., Michel, A., Parello, F., Treuil, M., and Valenza, M. (2000). Mobility and fluxes of major, minor and trace metals during basalt weathering and groundwater transport at Mt. Etna volcano (Sicily). *Geochimica et Cosmochimica Acta* 64, 1827-1841.
- Alloway, B.J. (2013). "Sources of heavy metals and metalloids in soils," in *Heavy metals in soils*. Springer), 11-50.
- Alonso, J.L., Mascellaro, S., Moreno, Y., Ferrús, M.A., and Hernández, J. (2002). Double-staining method for differentiation of morphological changes and membrane integrity of *Campylobacter coli* cells. *Appl Environ Microbiol* 68, 5151-5154.
- Altermann, W., Kazmierczak, J., Oren, A., and Wright, D. (2006). Cyanobacterial calcification and its rock-building potential during 3.5 billion years of Earth history. *Geobiology* 4, 147-166.
- Álvarez, B., López, M.M., and Biosca, E.G. (2008). Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. *Microbiology* 154, 3590-3598.
- Amor, K.B., Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, A.D., De Vos, W.M., and Abee, T. (2002a). Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. *J Appl Environ Microbiol* 68, 5209-5216.
- Amor, K.B., Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, A.D., De Vos, W.M., and Abee, T. (2002b). Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. *Applied and Environmental Microbiology* 68, 5209-5216.
- Anand, M., Crawford, I.A., Balat-Pichelin, M., Abanades, S., Van Westrenen, W., Péraudeau, G., Jaumann, R., and Seboldt, W. (2012). A brief review of chemical and mineralogical resources on the Moon and likely initial In Situ Resource Utilization (ISRU) applications. *Planetary and Space Science* 74, 42-48.
- Anderson, A.J., and Dawes, E.A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological reviews* 54, 450-472.
- Anderson, R.T., Chapelle, F.H., and Lovley, D.R. (1998). Evidence against hydrogen-based microbial ecosystems in basalt aquifers. *Science* 281, 976-977.
- Aneja, P., Dai, M., Lacorre, D.A., Pillon, B., and Charles, T.C. (2004). Heterologous complementation of the exopolysaccharide synthesis and carbon utilization phenotypes of *Sinorhizobium meliloti* Rm1021 polyhydroxyalkanoate synthesis mutants. *FEMS microbiology letters* 239, 277-283.
- Antony, C.P., Cockell, C.S., and Shouche, Y.S. (2012). Life in (and on) the rocks. *Journal of biosciences* 37, 3-11.
- Arku, B., Fanning, S., and Jordan, K. (2011). Flow cytometry to assess biochemical pathways in heat-stressed *Cronobacter* spp. (formerly *Enterobacter sakazakii*). *J Appl Microbiol* 111, 616-624.
- Arnalds, O. (2015). *The soils of Iceland*. Springer.

- Arnould, J., and Debus, A. (2008). An ethical approach to planetary protection. *Advances in Space Research* 42, 1089-1095.
- Astronoo (2014). *The roadmap for Mars mission* [Online]. Available: <http://www.astronoo.com/en/articles/living-on-mars.html> [Accessed 11-06-2017].
- Athanase, C.R., Steventenywa, J., Ebanyat, P., Makooma, M.T., Mukuralinda, A., and Nduwumuremyi, A. (2013). Phosphate sorption characteristics of andosols of the volcanic highlands of Central African Great Lakes Region. *Journal of Environmental Science and Engineering. A* 2, 89.
- Aubrey, A., Cleaves, H.J., Chalmers, J.H., Skelley, A.M., Mathies, R.A., Grunthaner, F.J., Ehrenfreund, P., and Bada, J.L. (2006). Sulfate minerals and organic compounds on Mars. *Geology* 34, 357-360.
- Ayrapetyan, M., and Oliver, J.D. (2016). The viable but non-culturable state and its relevance in food safety. *Current Opinion in Food Science* 8, 127-133.
- Baatout, S., De Boever, P., and Mergeay, M. (2005). Temperature-induced changes in bacterial physiology as determined by flow cytometry. *Ann Microbiol* 55, 73-80.
- Bach, W., and Edwards, K.J. (2003). Iron and sulfide oxidation within the basaltic ocean crust: implications for chemolithoautotrophic microbial biomass production. *Geochimica et Cosmochimica Acta* 67, 3871-3887.
- Bailey, B., Templeton, A., Staudigel, H., and Tebo, B.M. (2009). Utilization of substrate components during basaltic glass colonization by *Pseudomonas* and *Shewanella* isolates. *Geomicrobiology Journal* 26, 648-656.
- Bailey, V.L., Fansler, S.J., Stegen, J.C., and Mccue, L.A. (2013). Linking microbial community structure to β -glucosidic function in soil aggregates. *The ISME journal* 7, 2044-2053.
- Baker, G., Smith, J.J., and Cowan, D.A. (2003). Review and re-analysis of domain-specific 16S primers. *Journal of microbiological methods* 55, 541-555.
- Baker, P.W., and Leff, L. (2004). The effect of simulated microgravity on bacteria from the Mir space station. *Microgravity-Science and Technology* 15, 35-41.
- Banfield, J.F., Barker, W.W., Welch, S.A., and Taunton, A. (1999). Biological impact on mineral dissolution: application of the lichen model to understanding mineral weathering in the rhizosphere. *Proceedings of the National Academy of Sciences* 96, 3404-3411.
- Baranyi, J., and Roberts, T.A. (1994). A dynamic approach to predicting bacterial growth in food. *International journal of food microbiology* 23, 277-294.
- Barker, W., and Banfield, J. (1998). Zones of chemical and physical interaction at interfaces between microbial communities and minerals: a model. *Geomicrobiology Journal* 15, 223-244.
- Barker, W., Welch, S., Chu, S., and Banfield, J. (1998). Experimental observations of the effects of bacteria on aluminosilicate weathering. *American Mineralogist* 83, 1551-1563.
- Barker, W.W., Welch, S.A., and Banfield, J.F. (1997). Biogeochemical weathering of silicate minerals. *Reviews in Mineralogy and Geochemistry* 35, 391-428.
- Bates, R.L., and Jackson, J.A. (1987). Glossary of Geology: American Geological Institute. Alexandria, Virginia 788.
- Bell, J. (2008). *The Martian Surface: Composition, Mineralogy and Physical Properties*. Cambridge: Cambridge University Press.
- Benincasa, M., Pacor, S., Gennaro, R., and Scocchi, M. (2009). Rapid and reliable detection of antimicrobial peptide penetration into gram-negative bacteria based on fluorescence quenching. *Antimicrob Agents Chemother* 53, 3501-3504.
- Benner, S.A., Devine, K.G., Matveeva, L.N., and Powell, D.H. (2000). The missing organic molecules on Mars. *Proceedings of the National Academy of Sciences* 97, 2425-2430.
- Bennett, P., Rogers, J., Choi, W., and Hiebert, F. (2001). Silicates, silicate weathering, and microbial ecology. *Geomicrobiology Journal* 18, 3-19.

- Benoit, M.R., Conant, C.G., Ionescu-Zanetti, C., Schwartz, M., and Matin, A. (2010). New device for high-throughput viability screening of flow biofilms. *Appl Environ Microbiol* 76, 4136-4142.
- Benoit, M.R., and Klaus, D.M. (2007). Microgravity, bacteria, and the influence of motility. *Advances in Space Research* 39, 1225-1232.
- Benzerara, K., Barakat, M., Menguy, N., Guyot, F., De Luca, G., Audrain, C., and Heulin, T. (2004). Experimental colonization and alteration of orthopyroxene by the pleomorphic bacteria *Ramlibacter tataouinensis*. *Geomicrobiology Journal* 21, 341-349.
- Benzerara, K., Yoon, T.H., Menguy, N., Tyliczszak, T., and Brown, G.E. (2005). Nanoscale environments associated with bioweathering of a Mg-Fe-pyroxene. *Proceedings of the National Academy of Sciences of the United States of America* 102, 979-982.
- Bergen, B., Herlemann, D.P., Labrenz, M., and Jürgens, K. (2014). Distribution of the verrucomicrobial clade Spartobacteria along a salinity gradient in the Baltic Sea. *Environmental microbiology reports* 6, 625-630.
- Berner, E.K., and Berner, R.A. (2012). *Global environment: water, air, and geochemical cycles*. Princeton University Press.
- Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-U., and Egli, T. (2007). Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. *Applied and environmental microbiology* 73, 3283-3290.
- Berney, M., Vital, M., Huelshoff, I., Weilenmann, H.U., Egli, T., and Hammes, F. (2009). Rapid, cultivation-independent assessment of microbial viability in drinking water (vol 42, pg 4010, 2008). *Water Research* 43, 2567-2567.
- Berney, M., Vital, M., Hülshoff, I., Weilenmann, H.-U., Egli, T., and Hammes, F. (2008). Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Research* 42, 4010-4018.
- Beun, J., Dircks, K., Van Loosdrecht, M., and Heijnen, J. (2002). Poly- β -hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. *Water Research* 36, 1167-1180.
- Beveridge, T., and Murray, R. (1980). Sites of metal deposition in the cell wall of *Bacillus subtilis*. *Journal of bacteriology* 141, 876-887.
- Bibring, J.-P., Langevin, Y., Mustard, J.F., Poulet, F., Arvidson, R., Gendrin, A., Gondet, B., Mangold, N., Pinet, P., and Forget, F. (2006). Global mineralogical and aqueous Mars history derived from OMEGA/Mars Express data. *science* 312, 400-404.
- Bjelland, T., Grube, M., Hoem, S., Jorgensen, S.L., Daae, F.L., Thorseth, I.H., and Øvreås, L. (2011). Microbial metacommunities in the lichen–rock habitat. *Environmental microbiology reports* 3, 434-442.
- Bogosian, G., and Bourneuf, E.V. (2001). A matter of bacterial life and death. *EMBO reports* 2, 770-774.
- Boi, P., Amalfitano, S., Manti, A., Semprucci, F., Sisti, D., Rocchi, M., Balsamo, M., and Papa, S. (2015). Strategies for Water Quality Assessment: A Multiparametric Analysis of Microbiological Changes in River Waters. *River Res Appl*.
- Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., and Caporaso, J.G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature methods* 10, 57-59.
- Boston, P.J. (2010). Location, location, location! Lava caves on Mars for habitat, resources, and the search for life. *Journal of Cosmology* 12, 3957-3979.
- Boston, P.J., Ivanov, M.V., and McKay, C.P. (1992). On the possibility of chemosynthetic ecosystems in subsurface habitats on Mars. *Icarus* 95, 300-308.
- Bouix, M., and Ghorbal, S. (2015). Rapid assessment of *Oenococcus oeni* activity by measuring intracellular pH and membrane potential by flow cytometry, and its application to the more effective control of malolactic fermentation. *Int J Food Microbiol* 193, 139-146.

- Boult, S., Hand, V.L., and Vaughan, D.J. (2006). Microbial controls on metal mobility under the low nutrient fluxes found throughout the subsurface. *Science of the Total Environment* 372, 299-305.
- Brauer, H. (1990). Growth of fungi and bacteria in the reciprocating jet bioreactor. *Bioprocess and Biosystems Engineering* 6, 1-15.
- Breeuwer, P., and Abee, T. (2000a). Assessment of viability of microorganisms employing fluorescence techniques. *International journal of food microbiology* 55, 193-200.
- Breeuwer, P., and Abee, T. (2000b). Assessment of viability of microorganisms employing fluorescence techniques. *Int J Food Microbiol* 55, 193-200.
- Breeuwer, P., Drocourt, J.L., Rombouts, F.M., and Abee, T. (1996). A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester. *Appl Environ Microbiol* 62, 178-183.
- Brierley, J., and Brierley, C. (2001). Present and future commercial applications of biohydrometallurgy. *Hydrometallurgy* 59, 233-239.
- Brigham, C.J., Budde, C.F., Holder, J.W., Zeng, Q., Mahan, A.E., Rha, C., and Sinskey, A.J. (2010). Elucidation of β -oxidation pathways in *Ralstonia eutropha* H16 by examination of global gene expression. *Journal of bacteriology* 192, 5454-5464.
- Brim, H., Heyndrickx, M., De Vos, P., Wilmotte, A., Springael, D., Schlegel, H.G., and Mergeay, M. (1999). Amplified rDNA restriction analysis and further genotypic characterisation of metal-resistant soil bacteria and related facultative hydrogenotrophs. *Systematic and applied microbiology* 22, 258-268.
- Brognaux, A., Francis, F., Twizere, J.C., Thonart, P., and Delvigne, F. (2014). Scale-down effect on the extracellular proteome of *Escherichia coli*: correlation with membrane permeability and modulation according to substrate heterogeneities. *Bioprocess Biosyst Eng* 37, 1469-1485.
- Brown, R.B., Klaus, D., and Todd, P. (2002). Effects of space flight, clinorotation, and centrifugation on the substrate utilization efficiency of *E. coli*. *Microgravity science and technology* 13, 24-29.
- Bruno, L., Billi, D., Albertano, P., and Urzì, C. (2006). Genetic characterization of epilithic cyanobacteria and their associated bacteria. *Geomicrobiology Journal* 23, 293-299.
- Bryce, C., Byloos, B., Leys, N., and Cockell, C.S. (2016). Rock geochemistry induces stress and starvation responses in the bacterial proteome. *Environmental microbiology*.
- Brydie, J., Wogelius, R., Boult, S., Merrifield, C., and Vaughan, D. (2009). Model system studies of the influence of bacterial biofilm formation on mineral surface reactivity. *Biofouling* 25, 463-472.
- Budde, C.F., Riedel, S.L., Willis, L.B., Rha, C., and Sinskey, A.J. (2011). Production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. *Applied and environmental microbiology* 77, 2847-2854.
- Büdel, B. (1999). Ecology and diversity of rock-inhabiting cyanobacteria in tropical regions. *European Journal of Phycology* 34, 361-370.
- Bullen, T.D., and Eisenhauer, A. (2009). Metal stable isotopes in low-temperature systems: A primer. *Elements* 5, 349-352.
- Bunthof, C.J., and Abee, T. (2002). Development of a Flow Cytometric Method To Analyze Subpopulations of Bacteria in Probiotic Products and Dairy Starters. *Appl Environ Microbiol* 68, 2934-2942.
- Bunthof, C.J., Van Den Braak, S., Breeuwer, P., Rombouts, F.M., and Abee, T. (1999a). Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*. *Applied and Environmental Microbiology* 65, 3681-3689.
- Bunthof, C.J., Van Den Braak, S., Breeuwer, P., Rombouts, F.M., and Abee, T. (1999b). Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*. *Appl Environ Microbiol* 65, 3681-3689.
- Burdige, D.J., Dhakar, S.P., and Nealson, K.H. (1992). Effects of manganese oxide mineralogy on microbial and chemical manganese reduction. *Geomicrobiology Journal* 10, 27-48.

- Busch, M. (2004). Profitable asteroid mining. *Journal of the British Interplanetary Society* 57, 301-305.
- Buysschaert, B., Byloos, B., Leys, N., Van Houdt, R., and Boon, N. (2016). Reevaluating multicolor flow cytometry to assess microbial viability. *Applied Microbiology and Biotechnology*, 1-15.
- Cangelosi, G.A., and Meschke, J.S. (2014). Dead or alive: molecular assessment of microbial viability. *Appl Environ Microbiol* 80, 5884-5891.
- Carpenter, J., Fisackerly, R., and Houdou, B. (2016). Establishing lunar resource viability. *Space Policy* 37, 52-57.
- Carr, M.H., and Head, J.W. (2010). Geologic history of Mars. *Earth and Planetary Science Letters* 294, 185-203.
- Carson, J.K., Campbell, L., Rooney, D., Clipson, N., and Gleeson, D.B. (2009). Minerals in soil select distinct bacterial communities in their microhabitats. *FEMS microbiology ecology* 67, 381-388.
- Caruso, P., Palomo, J.L., Bertolini, E., Álvarez, B., López, M.M., and Biosca, E.G. (2005). Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: recovery of stressed cells at low temperatures. *Applied and environmental microbiology* 71, 140-148.
- Certini, G., Campbell, C.D., and Edwards, A.C. (2004). Rock fragments in soil support a different microbial community from the fine earth. *Soil Biology and Biochemistry* 36, 1119-1128.
- Chalia, S., Baskar, S., Prasad, M., Baskar, R., and Ranjan, K. (2016). Biomineralization abilities of *Cupriavidus* strain and *Bacillus subtilis* strains in vitro isolated from speleothems, Rani cave, Chattisgarh, India. *Geomicrobiology Journal*, 00-00.
- Chan, Y., Lacap, D.C., Lau, M.C., Ha, K.Y., Warren-Rhodes, K.A., Cockell, C.S., Cowan, D.A., McKay, C.P., and Pointing, S.B. (2012). Hypolithic microbial communities: between a rock and a hard place. *Environmental microbiology* 14, 2272-2282.
- Chang, A.C., and Cohen, S.N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *Journal of bacteriology* 134, 1141-1156.
- Chapelle, F. (2001). *Ground-water microbiology and geochemistry*. John Wiley & Sons.
- Characklis, W.G., and Marshall, K.C. (1990). Biofilms.
- Chen, G.Z., Fray, D.J., and Farthing, T.W. (2000a). Direct electrochemical reduction of titanium dioxide to titanium in molten calcium chloride. *Nature* 407, 361-364.
- Chen, J., Blume, H.-P., and Beyer, L. (2000b). Weathering of rocks induced by lichen colonization—a review. *Catena* 39, 121-146.
- Chen, S., Cao, Y., Ferguson, L.R., Shu, Q., and Garg, S. (2012a). Flow cytometric assessment of the protectants for enhanced in vitro survival of probiotic lactic acid bacteria through simulated human gastro-intestinal stresses. *Applied microbiology and biotechnology* 95, 345-356.
- Chen, S., Cao, Y., Ferguson, L.R., Shu, Q., and Garg, S. (2012b). Flow cytometric assessment of the protectants for enhanced in vitro survival of probiotic lactic acid bacteria through simulated human gastro-intestinal stresses. *Appl Microbiol Biotechnol* 95, 345-356.
- Chen, Y.-C., Chen, L.-A., Chen, S.-J., Chang, M.-C., and Chen, T.-L. (2004). A modified osmotic shock for periplasmic release of a recombinant creatinase from *Escherichia coli*. *Biochem Eng J* 19, 211-215.
- Chien, C.-C., Hong, C.-C., Soo, P.-C., Wei, Y.-H., Chen, S.-Y., Cheng, M.-L., and Sun, Y.-M. (2010). Functional expression of phaCAB genes from *Cupriavidus taiwanensis* strain 184 in *Escherichia coli* for polyhydroxybutyrate production. *Applied biochemistry and biotechnology* 162, 2355-2364.
- Childers, S.E., Ciufo, S., and Lovley, D.R. (2002). *Geobacter metallireducens* accesses insoluble Fe (III) oxide by chemotaxis. *Nature* 416, 767-769.

- Chou, L., and Wollast, R. (1984). Study of the weathering of albite at room temperature and pressure with a fluidized bed reactor. *Geochimica et Cosmochimica Acta* 48, 2205-2217.
- Clifford, S.M., Lasue, J., Heggy, E., Boisson, J., McGovern, P., and Max, M.D. (2010). Depth of the Martian cryosphere: Revised estimates and implications for the existence and detection of subpermafrost groundwater. *Journal of Geophysical Research: Planets* 115, n/a-n/a.
- Cockell, C.S. (2010). Geomicrobiology beyond Earth: microbe–mineral interactions in space exploration and settlement. *Trends in microbiology* 18, 308-314.
- Cockell, C.S. (2011). Synthetic geomicrobiology: engineering microbe–mineral interactions for space exploration and settlement. *International Journal of Astrobiology* 10, 315-324.
- Cockell, C.S. (2014). Trajectories of Martian habitability. *Astrobiology* 14, 182-203.
- Cockell, C.S., Kelly, L., and Summers, S. (2011). "Microbiology of volcanic environments," in *Extremophiles Handbook*. Springer, 917-933.
- Cockell, C.S., Kelly, L.C., and Marteinson, V. (2013). Actinobacteria—an ancient phylum active in volcanic rock weathering. *Geomicrobiology Journal* 30, 706-720.
- Cockell, C.S., Olsson, K., Knowles, F., Kelly, L., Herrera, A., Thorsteinsson, T., and Marteinson, V. (2009). Bacteria in weathered basaltic glass, Iceland. *Geomicrobiology Journal* 26, 491-507.
- Cockell, C.S., Osinski, G.R., and Lee, P. (2003). The impact crater as a habitat: effects of impact processing of target materials. *Astrobiology* 3, 181-191.
- Cockell, C.S., and Stokes, M.D. (2004). Ecology: widespread colonization by polar hypoliths. *Nature* 431, 414-414.
- Cocolin, L., Manzano, M., Cantoni, C., and Comi, G. (2001). Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Applied and Environmental Microbiology* 67, 5113-5121.
- Colman, S.M. (1982). "Chemical weathering of basalts and andesites; evidence from weathering rinds". USGPO).
- Comas, J., and Vives-Rego, J. (1997a). Assessment of the effects of Gramicidin, Formaldehyde, and surfactants on *Escherichia coli* by flow cytometry using nucleic acid and membrane potential dyes. *Cytometry* 29, 58-64.
- Comas, J., and Vives-Rego, J. (1997b). Assessment of the effects of gramicidin, formaldehyde, and surfactants on *Escherichia coli* by flow cytometry using nucleic acid and membrane potential dyes. *Cytometry* 29, 58-64.
- Cousins, C.R., and Cockell, C.S. (2016). An ESA roadmap for geobiology in space exploration. *Acta Astronautica* 118, 286-295.
- Craig, D., and Loughnan, F. (1964). Chemical and mineralogical transformations accompanying the weathering of basic volcanic rocks from New South Wales. *Soil Research* 2, 218-234.
- Crawford, I.A. (2015). Lunar resources: A review. *Progress in Physical Geography* 39, 137-167.
- Crispim, C., and Gaylarde, C. (2005). Cyanobacteria and biodeterioration of cultural heritage: a review. *Microbial ecology* 49, 1-9.
- Croal, L.R., Gralnick, J.A., Malasarn, D., and Newman, D.K. (2004). The genetics of geochemistry. *Annu. Rev. Genet.* 38, 175-202.
- Cronin, U.P., and Wilkinson, M.G. (2008a). *Bacillus cereus* endospores exhibit a heterogeneous response to heat treatment and low-temperature storage. *Food Microbiol* 25, 235-243.
- Cronin, U.P., and Wilkinson, M.G. (2008b). Monitoring growth phase-related changes in phosphatidylcholine-specific phospholipase C production, adhesion properties and physiology of *Bacillus cereus* vegetative cells. *Journal of industrial microbiology & biotechnology* 35, 1695-1703.

- Da Silva, T.L., Reis, A., Kent, C.A., Kosseva, M., Roseiro, J.C., and Hewitt, C.J. (2005). Stress-induced physiological responses to starvation periods as well as glucose and lactose pulses in *Bacillus licheniformis* CCMI 1034 continuous aerobic fermentation processes as measured by multi-parameter flow cytometry. *Biochemical engineering journal* 24, 31-41.
- Dalwai, F., Spratt, D., and Pratten, J. (2006). Modeling shifts in microbial populations associated with health or disease. *Appl Environ Microbiol* 72, 3678-3684.
- Dartnell, L.R., Desorgher, L., Ward, J., and Coates, A. (2007). Modelling the surface and subsurface martian radiation environment: implications for astrobiology. *Geophysical research letters* 34.
- Daskalakis, M., Magoulas, A., Kotoulas, G., Catsikis, I., Bakolas, A., Karageorgis, A., Mavridou, A., Doulia, D., and Rigas, F. (2013). *Pseudomonas*, *Pantoea* and *Cupriavidus* isolates induce calcium carbonate precipitation for bioremediation of ornamental stone. *Journal of applied microbiology* 115, 409-423.
- Daux, V., Crovisier, J., Hemond, C., and Petit, J. (1994). Geochemical evolution of basaltic rocks subjected to weathering: fate of the major elements, rare earth elements, and thorium. *Geochimica et Cosmochimica Acta* 58, 4941-4954.
- Davey, H.M. (2011). Life, death, and in-between: meanings and methods in microbiology. *Appl Environ Microbiol* 77, 5571-5576.
- Davis, C. (2014). Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria. *J Microbiol Methods* 103, 9-17.
- De Gelder, J., Vandenabeele, P., De Boever, P., Mergeay, M., Moens, L., and De Vos, P. (2009). Raman spectroscopic analysis of *Cupriavidus metallidurans* LMG 1195 (CH34) cultured in low-shear microgravity conditions. *Microgravity Science and Technology* 21, 217-223.
- De La Torre, R., Sancho, L.G., Horneck, G., De Los Ríos, A., Wierzbos, J., Olsson-Francis, K., Cockell, C.S., Rettberg, P., Berger, T., and De Vera, J.-P.P. (2010). Survival of lichens and bacteria exposed to outer space conditions—Results of the Lithopanspermia experiments. *Icarus* 208, 735-748.
- De Muynck, W., De Belie, N., and Verstraete, W. (2010). Microbial carbonate precipitation in construction materials: a review. *Ecological Engineering* 36, 118-136.
- De Roy, K., Clement, L., Thas, O., Wang, Y., and Boon, N. (2012). Flow cytometry for fast microbial community fingerprinting. *water research* 46, 907-919.
- Decker, E.M. (2001). The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral streptococci. *Letters in applied microbiology* 33, 188-192.
- Degelau, A., Scheper, T., Bailey, J., and Guske, C. (1995). Fluorometric measurement of poly- β hydroxybutyrate in *Alcaligenes eutrophus* by flow cytometry and spectrofluorometry. *Applied microbiology and biotechnology* 42, 653-657.
- Dejong, J.T., Fritzges, M.B., and Nüsslein, K. (2006). Microbially induced cementation to control sand response to undrained shear. *Journal of Geotechnical and Geoenvironmental Engineering* 132, 1381-1392.
- Dessert, C., Dupré, B., Gaillardet, J., François, L.M., and Allegre, C.J. (2003). Basalt weathering laws and the impact of basalt weathering on the global carbon cycle. *Chemical Geology* 202, 257-273.
- Diaper, J.P., Tither, K., and Edwards, C. (1992). Rapid Assessment of Bacterial Viability by Flow-Cytometry. *Appl Environ Microbiol* 58, 268-272.
- Diels, L., Van Roy, S., Somers, K., Willems, I., Doyen, W., Mergeay, M., Springael, D., and Leysen, R. (1995). The use of bacteria immobilized in tubular membrane reactors for heavy metal recovery and degradation of chlorinated aromatics. *Journal of membrane science* 100, 249-258.
- Diels, L., Van Roy, S., Taghavi, S., and Van Houdt, R. (2009). From industrial sites to environmental applications with *Cupriavidus metallidurans*. *Antonie Van Leeuwenhoek* 96, 247-258.

- Díez, B., Bauer, K., and Bergman, B. (2007). Epilithic cyanobacterial communities of a marine tropical beach rock (Heron Island, Great Barrier Reef): diversity and diazotrophy. *Applied and environmental microbiology* 73, 3656-3668.
- Dong, H. (2010). Mineral-microbe interactions: a review. *Frontiers of Earth Science in China* 4, 127-147.
- Dove, P.M. (2010). The rise of skeletal biominerals. *Elements* 6, 37-42.
- Drysdale, A., Ewert, M., and Hanford, A. (2003). Life support approaches for Mars missions. *Advances in Space Research* 31, 51-61.
- Dufrène, Y.F. (2002). Atomic force microscopy, a powerful tool in microbiology. *Journal of bacteriology* 184, 5205-5213.
- Dunn, M.F. (1998). Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. *FEMS microbiology reviews* 22, 105-123.
- Dupré, B., Dessert, C., Oliva, P., Goddérès, Y., Viers, J., François, L., Millot, R., and Gaillardet, J. (2003). Rivers, chemical weathering and Earth's climate. *Comptes Rendus Geoscience* 335, 1141-1160.
- Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods* 10, 996-998.
- Edwards, C.S., Christensen, P., and Hamilton, V. (2008). Evidence for extensive olivine-rich basalt bedrock outcrops in Ganges and Eos chasmas, Mars. *Journal of Geophysical Research: Planets* 113.
- Edwards, K., and Bazylinski, D. (2008). Intracellular minerals and metal deposits in prokaryotes. *Geobiology* 6, 309-317.
- Edwards, K.J., Bach, W., and Mccollom, T.M. (2005). Geomicrobiology in oceanography: microbe–mineral interactions at and below the seafloor. *TRENDS in Microbiology* 13, 449-456.
- Edwards, K.J., Bach, W., Mccollom, T.M., and Rogers, D.R. (2004). Neutrophilic iron-oxidizing bacteria in the ocean: their habitats, diversity, and roles in mineral deposition, rock alteration, and biomass production in the deep-sea. *Geomicrobiology Journal* 21, 393-404.
- Eggleton, R.A., Foudoulis, C., and Varkevisser, D. (1987). Weathering of basalt: changes in rock chemistry and mineralogy. *Clays and Clay Minerals* 35, 161-169.
- Ehlmann, B.L., Berger, G., Mangold, N., Michalski, J.R., Catling, D.C., Ruff, S.W., Chassefière, E., Niles, P.B., Chevrier, V., and Poulet, F. (2013). Geochemical consequences of widespread clay mineral formation in Mars' ancient crust. *Space Science Reviews* 174, 329-364.
- Ehrlich, H.L. (1996). How microbes influence mineral growth and dissolution. *Chemical geology* 132, 5-9.
- Ehrlich, L., and Newman, D. (2009). Geomicrobiology, 5th. CRC Press, Taylor & Francis Group, New York.
- Gomez-Villalba, B., Calvo, C., Vilchez, R., Gonzalez-Lopez, J. and Rodelas, B. (2006) TGGE analysis of the diversity of ammonia-oxidizing and denitrifying bacteria in submerged filter biofilms for the treatment of urban wastewater, *Appl. Microbiol. Biotechnol* 72, 393-400.
- Einen, J., Kruber, C., Øvreås, L., Thorseth, I., and Torsvik, T. (2006). Microbial colonization and alteration of basaltic glass. *Biogeosciences Discussions* 3, 273-307.
- Epstein, S. (2013). The phenomenon of microbial uncultivability. *Curr Opin Microbiol* 16, 636-642.
- Ericsson, M., Hanstorp, D., Hagberg, P., Enger, J., and Nystrom, T. (2000). Sorting out bacterial viability with optical tweezers. *J Bacteriol* 182, 5551-5555.
- Erlacher, A., Cernava, T., Cardinale, M., Soh, J., Sensen, C.W., Grube, M., and Berg, G. (2015). Rhizobiales as functional and endosymbiotic members in the lichen symbiosis of *Lobaria pulmonaria* L. *Frontiers in microbiology* 6, 53.
- Falcioni, T., Papa, S., and Gasol, J.M. (2008). Evaluating the flow-cytometric nucleic acid double-staining protocol in realistic situations of planktonic bacterial death. *J Appl Environ Microbiol* 74, 1767-1779.

- Ferrieres, L., Hémerly, G., Nham, T., Guérout, A.-M., Mazel, D., Beloin, C., and Ghigo, J.-M. (2010). Silent mischief: bacteriophage Mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *Journal of bacteriology* 192, 6418-6427.
- Ferris, J.P. (2005). Mineral catalysis and prebiotic synthesis: montmorillonite-catalyzed formation of RNA. *Elements* 1, 145-149.
- Fisk, M., Popa, R., Mason, O., Storrie-Lombardi, M., and Vicenzi, E. (2006). Iron-magnesium silicate bioweathering on Earth (and Mars?). *Astrobiology* 6, 48-68.
- Fitzgerald, D.J., Stratford, M., Gasson, M.J., Ueckert, J., Bos, A., and Narbad, A. (2004). Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. *J Appl Microbiol* 97, 104-113.
- Forster, S., Snape, J.R., Lappin-Scott, H.M., and Porter, J. (2002a). Simultaneous Fluorescent Gram Staining and Activity Assessment of Activated Sludge Bacteria. *Applied and Environmental Microbiology* 68, 4772-4779.
- Forster, S., Snape, J.R., Lappin-Scott, H.M., and Porter, J. (2002b). Simultaneous Fluorescent Gram Staining and Activity Assessment of Activated Sludge Bacteria. *Appl Environ Microbiol* 68, 4772-4779.
- Fouke, B.W., Bonheyo, G.T., Sanzenbacher, B., and Frias-Lopez, J. (2003). Partitioning of bacterial communities between travertine depositional facies at Mammoth Hot Springs, Yellowstone National Park, USA. *Canadian Journal of Earth Sciences* 40, 1531-1548.
- Frankel, R.B., and Bazylinski, D.A. (2003). Biologically induced mineralization by bacteria. *Reviews in mineralogy and geochemistry* 54, 95-114.
- Franzosa, E.A., Hsu, T., Sirota-Madi, A., Shafquat, A., Abu-Ali, G., Morgan, X.C., and Huttenhower, C. (2015). Sequencing and beyond: integrating molecular 'omics' for microbial community profiling. *Nature Reviews Microbiology* 13, 360-372.
- Frey, B., Rieder, S.R., Brunner, I., Plötze, M., Koetzsch, S., Lapanje, A., Brandl, H., and Furrer, G. (2010). Weathering-associated bacteria from the Damma glacier forefield: physiological capabilities and impact on granite dissolution. *Applied and environmental microbiology* 76, 4788-4796.
- Fridlund, M., and Kaltenegger, L. (2008). Mission requirements: how to search for extrasolar planets. *Extrasolar Planets*, S 51.
- Friedmann, E., Kappen, L., Meyer, M., and Nienow, J. (1993). Long-term productivity in the cryptoendolithic microbial community of the Ross Desert, Antarctica. *Microbial Ecology* 25, 51-69.
- Friedmann, E.I. (1980). "Endolithic microbial life in hot and cold deserts," in *Limits of Life*. Springer, 33-45.
- Frossard, E., Brossard, M., Hedley, M.J., and Metherell, A. (1995). Phosphorus in the global environment: transfers, cycles and management.
- Frost, B.R., and Frost, C.D. (2013). *Essentials of igneous and metamorphic petrology*. Cambridge University Press.
- Fujimura, R., Sato, Y., Nishizawa, T., Nanba, K., Oshima, K., Hattori, M., Kamijo, T., and Ohta, H. (2012). Analysis of early bacterial communities on volcanic deposits on the island of Miyake (Miyake-jima), Japan: a 6-year study at a fixed site. *Microbes and Environments* 27, 19-29.
- Fukuda, T., Fukuda, K., Takahashi, A., Ohnishi, T., Nakano, T., Sato, M., and Gunge, N. (2000). Analysis of deletion mutations of the rpsL gene in the yeast *Saccharomyces cerevisiae* detected after long-term flight on the Russian space station Mir. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 470, 125-132.
- Fuller, M.E., Streger, S.H., Rothmel, R.K., Mailloux, B.J., Hall, J.A., Onstott, T.C., Fredrickson, J.K., Balkwill, D.L., and DeFlaun, M.F. (2000). Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. *Appl Environ Microbiol* 66, 4486-4496.

- Furnes, H., Banerjee, N.R., Staudigel, H., and Muehlenbachs, K. (2007). Pillow lavas as a habitat for microbial life. *Geology Today* 23, 143-146.
- Gadd, G.M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156, 609-643.
- Gaillardet, J., Dupre, B., Allegre, C.J., and Négrel, P. (1997). Chemical and physical denudation in the Amazon River Basin. *Chemical geology* 142, 141-173.
- Garcia-Pichel, F. (2006). Plausible mechanisms for the boring on carbonates by microbial phototrophs. *Sedimentary Geology* 185, 205-213.
- Garrels, R.M., and Mackenzie, F.T. (1971). Evolution of sedimentary rocks.
- Gasol, J.M., and Del Giorgio, P.A. (2000). Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci Mar* 64, 197-224.
- Gauthier, D., Søbjerg, L.S., Jensen, K.M., Lindhardt, A.T., Bunge, M., Finster, K., Meyer, R.L., and Skrydstrup, T. (2010). Environmentally benign recovery and reactivation of palladium from industrial waste by using gram-negative bacteria. *ChemSusChem* 3, 1036-1039.
- Gislason, S.R., and Oelkers, E.H. (2003). Mechanism, rates, and consequences of basaltic glass dissolution: II. An experimental study of the dissolution rates of basaltic glass as a function of pH and temperature. *Geochimica et Cosmochimica Acta* 67, 3817-3832.
- Gitelson, J.I. (1992). Biological life-support systems for Mars mission. *Advances in Space Research* 12, 167-192.
- Gleeson, D., McDermott, F., and Clipson, N. (2006). Structural diversity of bacterial communities in a heavy metal mineralized granite outcrop. *Environmental microbiology* 8, 383-393.
- Gomes, A., Fernandes, E., and Lima, J.L. (2005). Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods* 65, 45-80.
- Gomez-Alvarez, V., King, G.M., and Nüsslein, K. (2007). Comparative bacterial diversity in recent Hawaiian volcanic deposits of different ages. *FEMS microbiology ecology* 60, 60-73.
- Goossens, O., Vanhavere, F., Leys, N., De Boever, P., O'sullivan, D., Zhou, D., Spurny, F., Yukihara, E., Gaza, R., and Mckeever, S. (2006). Radiation dosimetry for microbial experiments in the International Space Station using different etched track and luminescent detectors. *Radiation protection dosimetry* 120, 433-437.
- Gorbushina, A.A. (2007). Life on the rocks. *Environmental microbiology* 9, 1613-1631.
- Gorbushina, A.A., and Broughton, W.J. (2009). Microbiology of the atmosphere-rock interface: how biological interactions and physical stresses modulate a sophisticated microbial ecosystem. *Annual review of microbiology* 63, 431-450.
- Gorenflo, V., Steinbuchel, A., Marose, S., Rieseberg, M., and Scheper, T. (1999). Quantification of bacterial polyhydroxyalkanoic acids by Nile red staining. *Appl Microbiol Biotechnol* 51, 765-772.
- Goris, J., De Vos, P., Coenye, T., Hoste, B., Janssens, D., Brim, H., Diels, L., Mergeay, M., Kersters, K., and Vandamme, P. (2001). Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov. and *Ralstonia basilensis* Steinle et al. 1998 emend. *International journal of systematic and evolutionary microbiology* 51, 1773-1782.
- Greenspan, P., and Fowler, S.D. (1985). Spectrofluorometric studies of the lipid probe, Nile red. *J Lipid Res* 26, 781-789.
- Grey, B.E., and Steck, T.R. (2001). The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. *Applied and environmental microbiology* 67, 3866-3872.
- Griffiths, R.I., Whiteley, A.S., O'donnell, A.G., and Bailey, M.J. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA-and rRNA-based microbial community composition. *Applied and environmental microbiology* 66, 5488-5491.

- Gronstal, A., Pearson, V., Kappler, A., Dooris, C., Anand, M., Poitrasson, F., Kee, T.P., and Cockell, C.S. (2009). Laboratory experiments on the weathering of iron meteorites and carbonaceous chondrites by iron-oxidizing bacteria. *Meteoritics & Planetary Science* 44, 233-247.
- Gu, J.-D., Roman, M., Esselman, T., and Mitchell, R. (1998). The role of microbial biofilms in deterioration of space station candidate materials. *International biodeterioration & biodegradation* 41, 25-33.
- Gudbrandsson, S., Wolff-Boenisch, D., Gislason, S., and Oelkers, E. (2008). Dissolution rates of crystalline basalt at pH 4 and 10 and 25–75°C. *Mineralogical Magazine* 72, 155-158.
- Gudbrandsson, S., Wolff-Boenisch, D., Gislason, S.R., and Oelkers, E.H. (2011). An experimental study of crystalline basalt dissolution from $2 \leq \text{pH} \leq 11$ and temperatures from 5 to 75° C. *Geochimica et Cosmochimica Acta* 75, 5496-5509.
- Hall, K., Lindgren, B.S., and Jackson, P. (2005). Rock albedo and monitoring of thermal conditions in respect of weathering: some expected and some unexpected results. *Earth Surface Processes and Landforms* 30, 801-812.
- Hammes, F., Berney, M., and Egli, T. (2011). Cultivation-independent assessment of bacterial viability. *Adv Biochem Eng Biotechnol* 124, 123-150.
- Hammes, F., Broger, T., Weilenmann, H.U., Vital, M., Helbing, J., Bosshart, U., Huber, P., Odermatt, R.P., and Sonleitner, B. (2012). Development and laboratory-scale testing of a fully automated online flow cytometer for drinking water analysis. *Cytometry A* 81, 508-516.
- Hannig, C., Follo, M., Hellwig, E., and Al-Ahmad, A. (2010). Visualization of adherent micro-organisms using different techniques. *Journal of medical microbiology* 59, 1-7.
- Hansen, G., Johansen, C.L., Honore, A.H., Jensen, H.M., Jespersen, L., and Arneborg, N. (2015). Fluorescent labelling negatively affects the physiology of *Lactococcus lactis*. *Int Dairy J* 49, 130-138.
- Harrison, J., Turner, R., Marques, L., and Ceri, H. (2005a). A new understanding of these microbial communities is driving a revolution that may transform the science of microbiology. *Am Sci* 93, 508-515.
- Harrison, J.J., Turner, R.J., and Ceri, H. (2005b). Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*. *Environmental Microbiology* 7, 981-994.
- Haskin, L., and Warren, P. (1991). Lunar chemistry. *Lunar sourcebook* 4, 357-474.
- Hassler, D.M., Zeitlin, C., Wimmer-Schweingruber, R.F., Ehresmann, B., Rafkin, S., Eigenbrode, J.L., Brinza, D.E., Weigle, G., Böttcher, S., and Böhm, E. (2014). Mars' surface radiation environment measured with the Mars Science Laboratory's Curiosity rover. *science* 343, 1244797.
- Hastie, A.R., Kerr, A.C., Mitchell, S.F., and Millar, I.L. (2009). Geochemistry and tectonomagmatic significance of lower Cretaceous island arc lavas from the Devils Racecourse Formation, eastern Jamaica. *Geological Society, London, Special Publications* 328, 339-360.
- Hastie, A.R., Kerr, A.C., Pearce, J.A., and Mitchell, S. (2007). Classification of altered volcanic island arc rocks using immobile trace elements: development of the Th–Co discrimination diagram. *Journal of petrology* 48, 2341-2357.
- Hastings, J., and Wilson, T. (1976). Bioluminescence and chemiluminescence. *Photochem Photobiol* 23, 461-473.
- Hausrath, E., Neaman, A., and Brantley, S. (2009). Elemental release rates from dissolving basalt and granite with and without organic ligands. *American Journal of Science* 309, 633-660.
- Hazen, R.M., Papineau, D., Bleeker, W., Downs, R.T., Ferry, J.M., McCoy, T.J., Sverjensky, D.A., and Yang, H. (2008). Review Paper. Mineral evolution. *American Mineralogist* 93, 1693-1720.
- Heiken, G., Vaniman, D., and French, B.M. (1991). *Lunar sourcebook: A user's guide to the Moon*. CUP Archive.

- Heiken, G., and Vaniman, D.T. (Year). "Characterization of lunar ilmenite resources", in: *Lunar and Planetary Science Conference Proceedings*, 239-247.
- Hempel, F., Bozarth, A.S., Lindenkamp, N., Klingl, A., Zauner, S., Linne, U., Steinbüchel, A., and Maier, U.G. (2011). Microalgae as bioreactors for bioplastic production. *Microbial Cell Factories* 10, 81.
- Hendrickx, L., and Mergeay, M. (2007). From the deep sea to the stars: human life support through minimal communities. *Current opinion in microbiology* 10, 231-237.
- Henri, P.A., Rommevaux-Jestin, C., Lesongeur, F., Mumford, A., Emerson, D., Godfroy, A., and Ménez, B. (2015). Structural iron (II) of basaltic glass as an energy source for Zetaproteobacteria in an abyssal plain environment, off the Mid Atlantic Ridge. *Frontiers in microbiology* 6.
- Hepp, A.F., Landis, G.A., and Kubiak, C.P. (1993). A chemical approach to carbon dioxide utilization on Mars. *Resources of near-earth space*, 799-818.
- Herrera, A., and Cockell, C.S. (2007). Exploring microbial diversity in volcanic environments: a review of methods in DNA extraction. *Journal of Microbiological Methods* 70, 1-12.
- Herrera, A., Cockell, C.S., Self, S., Blaxter, M., Reitner, J., Arp, G., Dröse, W., Thorsteinsson, T., and Tindle, A.G. (2008). Bacterial colonization and weathering of terrestrial obsidian in Iceland. *Geomicrobiology Journal* 25, 25-37.
- Herrera, A., Cockell, C.S., Self, S., Blaxter, M., Reitner, J., Thorsteinsson, T., Arp, G., Dröse, W., and Tindle, A.G. (2009). A cryptoendolithic community in volcanic glass. *Astrobiology* 9, 369-381.
- Herrera, G., Martinez, A., Blanco, M., and O'connor, J.E. (2002). Assessment of *Escherichia coli* B with enhanced permeability to fluorochromes for flow cytometric assays of bacterial cell function. *Cytometry* 49, 62-69.
- Hewitt, C.J., Nebe-Von Caron, G., Nienow, A.W., and Mcfarlane, C.M. (1999). Use of multi-staining flow cytometry to characterise the physiological state of *Escherichia coli* W3110 in high cell density fed-batch cultures. *Biotechnol Bioeng* 63, 705-711.
- Hewitt, C.J., and Nebe-Von-Caron, G. (2001). An industrial application of multiparameter flow cytometry: assessment of cell physiological state and its application to the study of microbial fermentations. *Cytometry* 44, 179-187.
- Hill, T.C., Walsh, K.A., Harris, J.A., and Moffett, B.F. (2003). Using ecological diversity measures with bacterial communities. *FEMS microbiology ecology* 43, 1-11.
- Hodges, R., Hoffman, J., and Johnson, F.S. (1974). The lunar atmosphere. *Icarus* 21, 415-426.
- Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., and Saint, C.P. (2003). A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. *Journal of Microbiological Methods* 52, 379-388.
- Holladay, J.D., Brooks, K.P., Wegeng, R., Hu, J., Sanders, J., and Baird, S. (2007). Microreactor development for Martian in situ propellant production. *Catalysis today* 120, 35-44.
- Holmes, D.S., Cárdenas, J.P., Valdés, J.H., Quatrini, R., Esparza, M., Osorio, H., Duarte, F., Lefimil, C., and Jedlicki, E. (Year). "Comparative genomics begins to unravel the ecophysiology of bioleaching", in: *Advanced Materials Research: Trans Tech Publ*), 143-150.
- Holmquist, L., and Kjelleberg, S. (1993). Changes in viability, respiratory activity and morphology of the marine *Vibrio* sp. strain S14 during starvation of individual nutrients and subsequent recovery. *FEMS microbiology ecology* 12, 215-223.
- Hornbaek, T., Dynesen, J., and Jakobsen, M. (2002). Use of fluorescence ratio imaging microscopy and flow cytometry for estimation of cell vitality for *Bacillus licheniformis*. *Fems Microbiol Lett* 215, 261-265.
- Horneck, G., Klaus, D.M., and Mancinelli, R.L. (2010). Space microbiology. *Microbiology and Molecular Biology Reviews* 74, 121-156.

- Horváth, G., Petrás, M., Szentesi, G., Fábrián, Á., Park, J.W., Vereb, G., and Szöllősi, J. (2005). Selecting the right fluorophores and flow cytometer for fluorescence resonance energy transfer measurements. *Cytometry A* 65, 148-157.
- Huang, W.E., Stoecker, K., Griffiths, R., Newbold, L., Daims, H., Whiteley, A.S., and Wagner, M. (2007). Raman-FISH: combining stable-isotope raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function. *Environmental Microbiology* 9, 1878-1889.
- Hughes, K.A., and Lawley, B. (2003). A novel Antarctic microbial endolithic community within gypsum crusts. *Environmental Microbiology* 5, 555-565.
- Hutchens, E. (2009). Microbial selectivity on mineral surfaces: possible implications for weathering processes. *Fungal Biology Reviews* 23, 115-121.
- Hutchens, E., Gleeson, D., Mcdermott, F., Miranda-Casoluengo, R., and Clipson, N. (2010). Meter-scale diversity of microbial communities on a weathered pegmatite granite outcrop in the Wicklow Mountains, Ireland; evidence for mineral induced selection? *Geomicrobiology Journal* 27, 1-14.
- Hyka, P., Zullig, T., Ruth, C., Looser, V., Meier, C., Klein, J., Melzoch, K., Meyer, H.P., Glieder, A., and Kovar, K. (2010). Combined use of fluorescent dyes and flow cytometry to quantify the physiological state of *Pichia pastoris* during the production of heterologous proteins in high-cell-density fed-batch cultures. *Appl Environ Microbiol* 76, 4486-4496.
- Hytonen, J., Haataja, S., and Finne, J. (2006). Use of flow cytometry for the adhesion analysis of *Streptococcus pyogenes* mutant strains to epithelial cells: investigation of the possible role of surface pullulanase and cysteine protease, and the transcriptional regulator Rgg. *BMC Microbiol* 6.
- Ibekwe, A.M., Kennedy, A.C., Halvorson, J.J., and Yang, C.-H. (2007). Characterization of developing microbial communities in Mount St. Helens pyroclastic substrate. *Soil Biology and Biochemistry* 39, 2496-2507.
- Ichimura, A., Zent, A., Quinn, R., Sanchez, M., and Taylor, L. (2012). Hydroxyl (OH) production on airless planetary bodies: Evidence from H⁺/D⁺ ion-beam experiments. *Earth and Planetary Science Letters* 345, 90-94.
- Ivarsson, M., Bengtson, S., Belivanova, V., Stampanoni, M., Marone, F., and Tehler, A. (2012). Fossilized fungi in subseafloor Eocene basalts. *Geology* 40, 163-166.
- James, B.W., Mauchline, W.S., Dennis, P.J., Keevil, C.W., and Wait, R. (1999). Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. *Applied and environmental microbiology* 65, 822-827.
- Jameson, D.M., Croney, J.C., and Moens, P.D. (2003). [1] Fluorescence: Basic concepts, practical aspects, and some anecdotes. *Methods Enzymol* 360, 1-43.
- Jánosi, I.M., Czirók, A., Silhavy, D., and Holczinger, A. (2002). Is bioconvection enhancing bacterial growth in quiescent environments? *Environmental microbiology* 4, 525-531.
- Janssen, P.J., Van Houdt, R., Moors, H., Monsieurs, P., Morin, N., Michaux, A., Benotmane, M.A., Leys, N., Vallaes, T., and Lapidus, A. (2010). The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. *PLoS One* 5, e10433.
- Jefferson, K.K. (2004). What drives bacteria to produce a biofilm? *FEMS microbiology letters* 236, 163-173.
- Jepras, R.I., Carter, J., Pearson, S.C., Paul, F.E., and Wilkinson, M.J. (1995). Development of a robust flow cytometric assay for determining numbers of viable bacteria. *Appl Environ Microbiol* 61, 2696-2701.
- Jerez, C.A. (2008). The use of genomics, proteomics and other OMICS technologies for the global understanding of biomining microorganisms. *Hydrometallurgy* 94, 162-169.
- Jiang, J., Zhao, Q., Zhang, J., Zhang, G., and Lee, D.-J. (2009). Electricity generation from bio-treatment of sewage sludge with microbial fuel cell. *Bioresource Technology* 100, 5808-5812.
- Johnson, D.B., Grail, B.M., and Hallberg, K.B. (2013). A new direction for biomining: extraction of metals by reductive dissolution of oxidized ores. *Minerals* 3, 49-58.

- Johnson, I., and Spence, M. (2010). *The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies*. Life Technologies Corporation.
- Jones, A.A., and Bennett, P.C. (2014). Mineral microniches control the diversity of subsurface microbial populations. *Geomicrobiology Journal* 31, 246-261.
- Jones, A.A., and Bennett, P.C. (2017). Mineral Ecology: surface specific colonization and geochemical drivers of biofilm accumulation, composition, and phylogeny. *Frontiers in Microbiology* 8.
- Jones, R.M., Britt-Compton, B., and Williams, P.A. (2003). The naphthalene catabolic (nag) genes of *Ralstonia* sp. strain U2 are an operon that is regulated by NagR, a LysR-type transcriptional regulator. *Journal of bacteriology* 185, 5847-5853.
- José, R., Goebel, B.M., Friedmann, E.I., and Pace, N.R. (2003). Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. *Applied and Environmental Microbiology* 69, 3858-3867.
- Josef, J.A., Fisk, M.R., and Giovannoni, S. (Year). "Peridotite Dis solution Rates in Microbial Enrichment Cultures", in: *Proc. Ocean Drilling Program, Sci. Res.*, 1-38.
- Jost, L. (2006). Entropy and diversity. *Oikos* 113, 363-375.
- Kading, B., and Straub, J. (2015). Utilizing in-situ resources and 3D printing structures for a manned Mars mission. *Acta Astronautica* 107, 317-326.
- Kadouri, D., Jurkevitch, E., Okon, Y., and Castro-Sowinski, S. (2005). Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Critical reviews in microbiology* 31, 55-67.
- Kahl, W.-A. (2015). "Essentials of Igneous and Metamorphic Petrology". Mineral Soc America).
- Kahre, M.A., Murphy, J.R., and Haberle, R.M. (2006). Modeling the Martian dust cycle and surface dust reservoirs with the NASA Ames general circulation model. *Journal of Geophysical Research: Planets* 111.
- Kalinowski, B.E., Liermann, L.J., Givens, S., and Brantley, S.L. (2000). Rates of bacteria-promoted solubilization of Fe from minerals: a review of problems and approaches. *Chemical geology* 169, 357-370.
- Kappler, A., and Straub, K.L. (2005). Geomicrobiological cycling of iron. *Reviews in Mineralogy and Geochemistry* 59, 85-108.
- Kawai, M., Yamaguchi, N., and Nasu, M. (1999). Rapid enumeration of physiologically active bacteria in purified water used in the pharmaceutical manufacturing process. *Journal of applied microbiology* 86, 496-504.
- Keer, J., and Birch, L. (2003). Molecular methods for the assessment of bacterial viability. *J Microbiol Methods* 53, 175-183.
- Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R., and Barer, M.R. (1998). Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* 73, 169-187.
- Kelly, L., Colin, Y., Turpault, M., and Uroz, S. (2016). Mineral Type and Solution Chemistry Affect the Structure and Composition of Actively Growing Bacterial Communities as Revealed by Bromodeoxyuridine Immunocapture and 16S rRNA Pyrosequencing. *Microbial ecology* 72, 428-442.
- Kelly, L.C., Cockell, C.S., Herrera-Belaroussi, A., Piceno, Y., Andersen, G., Desantis, T., Brodie, E., Thorsteinsson, T., Marteinson, V., and Poly, F. (2011). Bacterial diversity of terrestrial crystalline volcanic rocks, Iceland. *Microbial ecology* 62, 69-79.
- Kelly, L.C., Cockell, C.S., Piceno, Y.M., Andersen, G.L., Thorsteinsson, T., and Marteinson, V. (2010). Bacterial diversity of weathered terrestrial Icelandic volcanic glasses. *Microbial ecology* 60, 740-752.
- Kelly, L.C., Cockell, C.S., Thorsteinsson, T., Marteinson, V., and Stevenson, J. (2014). Pioneer microbial communities of the Fimmvörðuháls lava flow, Eyjafjallajökull, Iceland. *Microbial ecology* 68, 504-518.
- Kennedy, M., Chadwick, O., Vitousek, P., Derry, L., and Hendricks, D. (1998). Changing sources of base cations during ecosystem development, Hawaiian Islands. *Geology* 26, 1015-1018.

- Kim, J.S., Lee, K.C., Kim, D.S., Ko, S.H., Jung, M.Y., Rhee, S.K., and Lee, J.S. (2015). Pyrosequencing analysis of a bacterial community associated with lava-formed soil from the Gotjawal forest in Jeju, Korea. *MicrobiologyOpen* 4, 301-312.
- Kim, W., Tengra, F.K., Shong, J., Marchand, N., Chan, H.K., Young, Z., Pangule, R.C., Parra, M., Dordick, J.S., and Plawsky, J.L. (2013a). Effect of spaceflight on *Pseudomonas aeruginosa* final cell density is modulated by nutrient and oxygen availability. *BMC microbiology* 13, 241.
- Kim, W., Tengra, F.K., Young, Z., Shong, J., Marchand, N., Chan, H.K., Pangule, R.C., Parra, M., Dordick, J.S., and Plawsky, J.L. (2013b). Spaceflight promotes biofilm formation by *Pseudomonas aeruginosa*. *PloS one* 8, e62437.
- Kim, W., Tengra, F.K., Young, Z., Shong, J., Marchand, N., Chan, H.K., Pangule, R.C., Parra, M., Dordick, J.S., Plawsky, J.L., and Collins, C.H. (2013c). Spaceflight promotes biofilm formation by *Pseudomonas aeruginosa*. *PLoS One* 8, e62437.
- Kim, Y.-H., Bae, B., and Choung, Y.-K. (2005). Optimization of biological phosphorus removal from contaminated sediments with phosphate-solubilizing microorganisms. *Journal of bioscience and bioengineering* 99, 23-29.
- Kimble, J., Ping, C., Sumner, M., and Wilding, L. (2000). Andisols. *Handbook of soil science*, E209-E224.
- Klas, M., Tsafnat, N., Dennerley, J., Beckmann, S., Osborne, B., Dempster, A.G., and Manefield, M. (2015). Biomining and methanogenesis for resource extraction from asteroids. *Space Policy* 34, 18-22.
- Klaus, D., Simske, S., Todd, P., and Stodieck, L. (1997). Investigation of space flight effects on *Escherichia coli* and a proposed model of underlying physical mechanisms. *Microbiology* 143, 449-455.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glöckner, F.O. (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic acids research*, gks808.
- Kminek, G., and Rummel, J. (2015). COSPAR's planetary protection policy. *Space Research Today* 193, 7-19.
- Knelman, J.E., Legg, T.M., O'Neill, S.P., Washenberger, C.L., González, A., Cleveland, C.C., and Nemergut, D.R. (2012). Bacterial community structure and function change in association with colonizer plants during early primary succession in a glacier forefield. *Soil Biology and Biochemistry* 46, 172-180.
- Kobayashi, H., Oethinger, M., Tuohy, M.J., Procop, G.W., and Bauer, T.W. (2009). Improved detection of biofilm-formative bacteria by vortexing and sonication: a pilot study. *Clinical orthopaedics and related research* 467, 1360-1364.
- Koch, C., Gunther, S., Desta, A.F., Hubschmann, T., and Muller, S. (2013). Cytometric fingerprinting for analyzing microbial intracommunity structure variation and identifying subcommunity function. *Nat Protoc* 8, 190-202.
- Konhauser, K., Lalonde, S., and Phoenix, V. (2008). Bacterial biomineralization: Where to from here? *Geobiology* 6, 298-302.
- Konhauser, K.O., Amskold, L., Lalonde, S.V., Posth, N.R., Kappler, A., and Anbar, A. (2007). Decoupling photochemical Fe (II) oxidation from shallow-water BIF deposition. *Earth and Planetary Science Letters* 258, 87-100.
- Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental microbiology* 79, 5112-5120.
- Kraemer, S.M., Butler, A., Borer, P., and Cervini-Silva, J. (2005). Siderophores and the dissolution of iron-bearing minerals in marine systems. *Reviews in mineralogy and geochemistry* 59, 53-84.
- Kurtz, H., Cox, R., and Reisch, C. (2005). A microcosm system for the study of cryptoendolithic microbial biofilms from desert ecosystems. *Biofilms* 2, 145-152.

- Lahtinen, S.J., Ouwehand, A.C., Reinikainen, J.P., Korpela, J.M., Sandholm, J., and Salminen, S.J. (2006). Intrinsic properties of so-called dormant probiotic bacteria, determined by flow cytometric viability assays. *Appl Environ Microbiol* 72, 5132-5134.
- Lauber, C.L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and environmental microbiology* 75, 5111-5120.
- Lawrence, J., Neu, T., and Swerhone, G. (1998). Application of multiple parameter imaging for the quantification of algal, bacterial and exopolymer components of microbial biofilms. *J Microbiol Methods* 32, 253-261.
- Le Bas, M., Le Maitre, R., and Woolley, A. (1992). The construction of the total alkali-silica chemical classification of volcanic rocks. *Mineralogy and Petrology* 46, 1-22.
- Le Bas, M., and Streckeisen, A.L. (1991). The IUGS systematics of igneous rocks. *Journal of the Geological Society* 148, 825-833.
- Lebaron, P., Parthuisot, N., and Catala, P. (1998). Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Appl Environ Microbiol* 64, 1725-1730.
- Lechevalier, H.A., and Lechevalier, M.P. (1967). Biology of actinomycetes. *Annual Reviews in Microbiology* 21, 71-100.
- Ledrich, M.-L., Stemmler, S., Laval-Gilly, P., Foucaud, L., and Falla, J. (2005). Precipitation of silver-thiosulfate complex and immobilization of silver by *Cupriavidus metallidurans* CH34. *Biometals* 18, 643-650.
- Lee, M.D., Walworth, N.G., Sylvan, J.B., Edwards, K.J., and Orcutt, B.N. (2015). Microbial communities on seafloor basalts at Dorado Outcrop reflect level of alteration and highlight global lithic clades. *Frontiers in microbiology* 6, 1470.
- Lee, Y.K., Ho, P.S., Low, C.S., Arvilommi, H., and Salminen, S. (2004). Permanent colonization by *Lactobacillus casei* is hindered by the low rate of cell division in mouse gut. *Appl Environ Microbiol* 70, 670-674.
- Lehto, K., Kanervo, E., Stahle, K., Lehto, H., Tammi, M., and Virtanen, J. (2007). Photosynthetic life support systems in the Martian conditions. *ROME: Response of Organisms to the Martian Environment*, 151-160.
- Leroy, B., Rosier, C., Erculisse, V., Leys, N., Mergeay, M., and Wattiez, R. (2010). Differential proteomic analysis using isotope-coded protein-labeling strategies: comparison, improvements and application to simulated microgravity effect on *Cupriavidus metallidurans* CH34. *Proteomics* 10, 2281-2291.
- Leys, N., Baatout, S., Rosier, C., Dams, A., S'heeren, C., Wattiez, R., and Mergeay, M. (2009). The response of *Cupriavidus metallidurans* CH34 to spaceflight in the international space station. *Antonie Van Leeuwenhoek* 96, 227-245.
- Leys, N., Hendrickx, L., De Boever, P., Baatout, S., and Mergeay, M. (2004). Space flight effects on bacterial physiology. *Journal of biological regulators and homeostatic agents* 18, 193-199.
- Leyval, C., and Berthelin, J. (1989). Interactions between *Laccaria laccata*, *Agrobacterium radiobacter* and beech roots: Influence on P, K, Mg, and Fe mobilization from minerals and plant growth. *Plant and Soil* 117, 103-110.
- Li, L., Mendis, N., Trigui, H., Oliver, J.D., and Faucher, S.P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol* 5.
- Lian, B., Hu, Q., Chen, J., Ji, J., and Teng, H.H. (2006). Carbonate biomineralization induced by soil bacterium *Bacillus megaterium*. *Geochimica et Cosmochimica Acta* 70, 5522-5535.
- Lieder, S., Jahn, M., Koepff, J., Müller, S., and Takors, R. (2016). Environmental stress speeds up DNA replication in *Pseudomonas putida* in chemostat cultivations. *Biotechnol J* 11, 155-163.
- Lim, S., and Anand, M. (2015). In-Situ Resource Utilisation (ISRU) derived extra-terrestrial construction processes using sintering-based additive manufacturing techniques—focusing on a lunar surface environment.

- Linhova, M., Branska, B., Patakova, P., Lipovsky, J., Fribert, P., Rychtera, M., and Melzoch, K. (2012). Rapid flow cytometric method for viability determination of solventogenic clostridia. *Folia Microbiol* 57, 307-311.
- Liu, Y., Cockell, C.S., Wang, G., Hu, C., Chen, L., and De Philippis, R. (2008). Control of lunar and Martian dust—experimental insights from artificial and natural cyanobacterial and algal crusts in the desert of Inner Mongolia, China. *Astrobiology* 8, 75-86.
- Lopez-Amoros, R., Castel, S., Comas-Riu, J., and Vives-Rego, J. (1997). Assessment of *E. coli* and *Salmonella* viability and starvation by confocal laser microscopy and flow cytometry using rhodamine 123, DiBAC4(3), propidium iodide, and CTC. *Cytometry* 29, 298-305.
- Lopez-Amoros, R., Comas, J., and Vives-Rego, J. (1995). Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using Rhodamine 123, propidium iodide and oxonol. *Appl Environ Microbiol* 61, 2521-2526.
- López, N.I., Floccari, M.E., Steinbüchel, A., García, A.F., and Méndez, B.S. (1995). Effect of poly (3-hydroxybutyrate)(PHB) content on the starvation-survival of bacteria in natural waters. *FEMS microbiology ecology* 16, 95-101.
- López, N.I., Pettinari, M.J., Nikel, P.I., and Méndez, B.S. (2015). Chapter Three- Polyhydroxyalkanoates: Much More than Biodegradable Plastics. *Advances in applied microbiology* 93, 73-106.
- Loudon, C.-M., Nicholson, N., Finster, K., Leys, N., Byloos, B., Van Houdt, R., Rettberg, P., Moeller, R., Fuchs, F.M., Demets, R., Krause, J., Vukich, M., Mariani, A., and Cockell, C. (2017). BioRock: new experiments and hardware to investigate microbe–mineral interactions in space. *International Journal of Astrobiology*, 1-11.
- Loughnan, F.C. (1969). Chemical weathering of the silicate minerals.
- Louvat, P., and Allègre, C.J. (1997). Present denudation rates on the island of Reunion determined by river geochemistry: basalt weathering and mass budget between chemical and mechanical erosions. *Geochimica et Cosmochimica Acta* 61, 3645-3669.
- Lovley, D.R. (2000). "Fe (III) and Mn (IV) reduction," in *Environmental microbe-metal interactions*. American Society of Microbiology), 3-30.
- Lower, S.K., Tadanier, C.J., and Hochella, M.F. (2000). Measuring interfacial and adhesion forces between bacteria and mineral surfaces with biological force microscopy. *Geochimica et Cosmochimica Acta* 64, 3133-3139.
- Lozupone, C.A., and Knight, R. (2008). Species divergence and the measurement of microbial diversity. *FEMS microbiology reviews* 32, 557-578.
- Lukito, H.P., Kouno, K., and Ando, T. (1998). Phosphorus requirements of microbial biomass in a regosol and an andosol. *Soil Biology and Biochemistry* 30, 865-872.
- Lüttge, A., and Conrad, P.G. (2004). Direct observation of microbial inhibition of calcite dissolution. *Applied and Environmental Microbiology* 70, 1627-1632.
- Lysnes, K., Thorseth, I.H., Steinsbu, B.O., Øvreås, L., Torsvik, T., and Pedersen, R.B. (2004). Microbial community diversity in seafloor basalt from the Arctic spreading ridges. *FEMS Microbiology Ecology* 50, 213-230.
- Madigan, M.T., Martinko, J.M., Dunlap, P.V., and Clark, D.P. (2008). Brock Biology of microorganisms 12th edn. *Int. Microbiol* 11, 65-73.
- Maggi, F., and Pallud, C. (2010). Space agriculture in micro-and hypo-gravity: A comparative study of soil hydraulics and biogeochemistry in a cropping unit on Earth, Mars, the Moon and the space station. *Planetary and Space Science* 58, 1996-2007.
- Mah, T.-F., Pitts, B., Pellock, B., Walker, G.C., Stewart, P.S., and O'toole, G.A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nat* 426, 306-310.
- Mailloux, B.J., Alexandrova, E., Keimowitz, A.R., Wovkulich, K., Freyer, G.A., Herron, M., Stolz, J.F., Kenna, T.C., Pichler, T., and Polizzotto, M.L. (2009). Microbial mineral weathering for nutrient acquisition releases arsenic. *Applied and environmental microbiology* 75, 2558-2565.

- Malmcrona-Friberg, K., Tunlid, A., Mårdén, P., Kjelleberg, S., and Odham, G. (1986). Chemical changes in cell envelope and poly- β -hydroxybutyrate during short term starvation of a marine bacterial isolate. *Archives of microbiology* 144, 340-345.
- Malmström, M., and Banwart, S. (1997). Biotite dissolution at 25 C: The pH dependence of dissolution rate and stoichiometry. *Geochimica et Cosmochimica Acta* 61, 2779-2799.
- Manuel, C.M., Nunes, O.C., and Melo, L. (2007). Dynamics of drinking water biofilm in flow/non-flow conditions. *Water Research* 41, 551-562.
- Mapelli, F., Marasco, R., Balloi, A., Rolli, E., Cappitelli, F., Daffonchio, D., and Borin, S. (2012). Mineral-microbe interactions: biotechnological potential of bioweathering. *Journal of Biotechnology* 157, 473-481.
- Mapelli, F., Marasco, R., Rizzi, A., Baldi, F., Ventura, S., Daffonchio, D., and Borin, S. (2011). Bacterial communities involved in soil formation and plant establishment triggered by pyrite bioweathering on Arctic moraines. *Microbial ecology* 61, 438-447.
- Marie, D., Vaultot, D., and Partensky, F. (1996). Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and PicoGreen for flow cytometric analysis of marine prokaryotes. *Appl Environ Microbiol* 62, 1649-1655.
- Marshall, K. (1975). Clay mineralogy in relation to survival of soil bacteria. *Annual Review of Phytopathology* 13, 357-373.
- Marshall, K.C. (1988). Adhesion and growth of bacteria at surfaces in oligotrophic habitats. *Canadian journal of microbiology* 34, 503-506.
- Marshall, M.J., Beliaev, A.S., Dohnalkova, A.C., Kennedy, D.W., Shi, L., Wang, Z., Boyanov, M.I., Lai, B., Kemner, K.M., and Mclean, J.S. (2006). c-Type cytochrome-dependent formation of U (IV) nanoparticles by *Shewanella oneidensis*. *PLoS Biol* 4, e268.
- Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., and Bond, D.R. (2008). *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences* 105, 3968-3973.
- Martín-Torres, F.J., Zorzano, M.-P., Valentín-Serrano, P., Harri, A.-M., Genzer, M., Kemppinen, O., Rivera-Valentin, E.G., Jun, I., Wray, J., and Madsen, M.B. (2015). Transient liquid water and water activity at Gale crater on Mars. *Nature Geoscience* 8, 357-361.
- Mason, O.U., Di Meo-Savoie, C.A., Van Nostrand, J.D., Zhou, J., Fisk, M.R., and Giovannoni, S.J. (2009). Prokaryotic diversity, distribution, and insights into their role in biogeochemical cycling in marine basalts. *The ISME journal* 3, 231-242.
- Mason, O.U., Nakagawa, T., Rosner, M., Van Nostrand, J.D., Zhou, J., Maruyama, A., Fisk, M.R., and Giovannoni, S.J. (2010). First investigation of the microbiology of the deepest layer of ocean crust. *PLoS One* 5, e15399.
- Mastroleo, F., Van Houdt, R., Leroy, B., Benotmane, M.A., Janssen, A., Mergeay, M., Vanhavere, F., Hendrickx, L., Wattiez, R., and Leys, N. (2009). Experimental design and environmental parameters affect *Rhodospirillum rubrum* S1H response to space flight. *The ISME journal* 3, 1402-1419.
- Matos, C.T., and Da Silva, T.L. (2013). Using multi-parameter flow cytometry as a novel approach for physiological characterization of bacteria in microbial fuel cells. *Process Biochemistry* 48, 49-57.
- Mauck, B.S., and Roberts, J.A. (2007). Mineralogic control on abundance and diversity of surface-adherent microbial communities. *Geomicrobiology Journal* 24, 167-177.
- Maurice, P.A., Haack, E.A., and Mishra, B. (2009). Siderophore sorption to clays. *Biometals* 22, 649-658.
- Maurice, P.A., Vierkorn, M.A., Hersman, L.E., Fulghum, J.E., and Ferryman, A. (2001). Enhancement of kaolinite dissolution by an aerobic *Pseudomonas mendocina* bacterium. *Geomicrobiology Journal* 18, 21-35.
- Mautner, M.N. (2002). Planetary resources and astroecology. Planetary microcosm models of asteroid and meteorite interiors: electrolyte solutions and microbial growth—implications for space populations and panspermia. *Astrobiology* 2, 59-76.

- Mcalister, M., Kulakov, L., O'hanlon, J., Larkin, M., and Ogden, K.L. (2002). Survival and nutritional requirements of three bacteria isolated from ultrapure water. *Journal of industrial microbiology & biotechnology* 29, 75-82.
- Mcdaniel, P.A., Lowe, D.J., Arnalds, O., and Ping, C.-L. (2012). Andisols.
- Mcgrail, B.P., Schaef, H.T., Ho, A.M., Chien, Y.J., Dooley, J.J., and Davidson, C.L. (2006). Potential for carbon dioxide sequestration in flood basalts. *Journal of Geophysical Research: Solid Earth* 111.
- Mckay, C.P., and Friedmann, E.I. (1985). The cryptoendolithic microbial environment in the Antarctic cold desert: temperature variations in nature. *Polar Biology* 4, 19-25.
- Mckay, C.P., and Marinova, M.M. (2001). The physics, biology, and environmental ethics of making Mars habitable. *Astrobiology* 1, 89-109.
- Mckay, D.S., Heiken, G., Basu, A., Blanford, G., Simon, S., Reedy, R., French, B.M., and Papike, J. (1991). The lunar regolith. *Lunar sourcebook*, 285-356.
- Mclean, R.J., Cassanto, J.M., Barnes, M.B., and Koo, J.H. (2001). Bacterial biofilm formation under microgravity conditions. *FEMS Microbiology Letters* 195, 115-119.
- Mclennan, S.M. (2003). Sedimentary silica on Mars. *Geology* 31, 315-318.
- Mcloughlin, N., Brasier, M.D., Wacey, D., Green, O.R., and Perry, R.S. (2007). On biogenicity criteria for endolithic microborings on early Earth and beyond. *Astrobiology* 7, 10-26.
- Mcloughlin, N., Furnes, H., Banerjee, N., Muehlenbachs, K., and Staudigel, H. (2009). Ichnotaxonomy of microbial trace fossils in volcanic glass. *Journal of the Geological Society* 166, 159-169.
- Mcmahon, S., Parnell, J., Ponicka, J., Hole, M., and Boyce, A. (2013). The habitability of vesicles in martian basalt. *Astronomy and Geophysics* 54, 1.17-11.21.
- Mcsween, H., Arvidson, R., Bell, J., Blaney, D., Cabrol, N., Christensen, P., Clark, B., Crisp, J., Crumpler, L., and Des Marais, D. (2004). Basaltic rocks analyzed by the Spirit rover in Gusev Crater. *Science* 305, 842-845.
- Menezes, A.A., Cumbers, J., Hogan, J.A., and Arkin, A.P. (2015a). Towards synthetic biological approaches to resource utilization on space missions. *Journal of The Royal Society Interface* 12, 20140715.
- Menezes, A.A., Montague, M.G., Cumbers, J., Hogan, J.A., and Arkin, A.P. (2015b). Grand challenges in space synthetic biology. *Journal of The Royal Society Interface* 12, 20150803.
- Mergeay, M., Monchy, S., Vallaey, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., Van Der Lelie, D., and Wattiez, R. (2003). *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS microbiology reviews* 27, 385-410.
- Mergeay, M., Nies, D., Schlegel, H., Gerits, J., Charles, P., and Van Gijsegem, F. (1985). *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *Journal of bacteriology* 162, 328-334.
- Mergeay, M., and Van Houdt, R. (2014). "Adaptation to xenobiotics and toxic compounds by *Cupriavidus* and *Ralstonia* with special reference to *Cupriavidus metallidurans* CH34 and mobile genetic elements," in *Biodegradative Bacteria*. Springer), 105-127.
- Mergeay, M., and Van Houdt, R. (2015). *Metal Response in Cupriavidus metallidurans: Volume I: From Habitats to Genes and Proteins*. Springer.
- Meyerhoff, A.A., Taner, I., Morris, A., Agocs, W., Kamen-Kaye, M., Bhat, M., Smoot, N., Choi, D.R., and Hull, D.M. (1996). "Magma Floods, Flood Basalts, and Surge Tectonics," in *Surge Tectonics: A New Hypothesis of Global Geodynamics*. Springer), 192-254.
- Mijnendonckx, K., Provoost, A., Monsieurs, P., Leys, N., Mergeay, M., Mahillon, J., and Van Houdt, R. (2011). Insertion sequence elements in *Cupriavidus metallidurans* CH34: distribution and role in adaptation. *Plasmid* 65, 193-203.
- Mijnendonckx, K., Provoost, A., Ott, C., Venkateswaran, K., Mahillon, J., Leys, N., and Van Houdt, R. (2013). Characterization of the survival ability of *Cupriavidus metallidurans*

- and *Ralstonia pickettii* from space-related environments. *Microbial ecology* 65, 347-360.
- Miller, A.G., and Colman, B. (1980). Evidence for HCO₃⁻ transport by the blue-green alga (cyanobacterium) *Coccochloris peniocyctis*. *Plant Physiology* 65, 397-402.
- Ming, D., Archer, P., Glavin, D., Eigenbrode, J., Franz, H., Sutter, B., Brunner, A., Stern, J., Freissinet, C., and Mcadam, A. (2014). Volatile and organic compositions of sedimentary rocks in Yellowknife Bay, Gale Crater, Mars. *Science* 343, 1245267.
- Miot, J., Benzerara, K., and Kappler, A. (2014). Investigating microbe-mineral interactions: Recent advances in X-ray and electron microscopy and redox-sensitive methods. *Annual Review of Earth and Planetary Sciences* 42, 271-289.
- Mitchell, A.C., Lafrenière, M.J., Skidmore, M.L., and Boyd, E.S. (2013). Influence of bedrock mineral composition on microbial diversity in a subglacial environment. *Geology* 41, 855-858.
- Möllers, K.B., Cannella, D., Jørgensen, H., and Frigaard, N.-U. (2014). Cyanobacterial biomass as carbohydrate and nutrient feedstock for bioethanol production by yeast fermentation. *Biotechnology for biofuels* 7, 64.
- Monsieurs, P., Moors, H., Van Houdt, R., Janssen, P.J., Janssen, A., Coninx, I., Mergeay, M., and Leys, N. (2011). Heavy metal resistance in *Cupriavidus metallidurans* CH34 is governed by an intricate transcriptional network. *Biometals* 24, 1133-1151.
- Montague, M., McArthur IV, G.H., Cockell, C.S., Held, J., Marshall, W., Sherman, L.A., Wang, N., Nicholson, W.L., Tarjan, D.R., and Cumbers, J. (2012). "The role of synthetic biology for in situ resource utilization (ISRU)". Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA).
- Moskovitz, N.A., Jedicke, R., Gaidos, E., Willman, M., Nesvorný, D., Fevig, R., and Ivezić, Ž. (2008). The distribution of basaltic asteroids in the Main Belt. *Icarus* 198, 77-90.
- Muller, S. (2007). Modes of cytometric bacterial DNA pattern: a tool for pursuing growth. *Cell Prolif* 40, 621-639.
- Muller, S., and Nebe-Von-Caron, G. (2010). Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. *FEMS Microbiol Rev* 34, 554-587.
- Munzel, T., Afanas'ev, I.B., Kleschyov, A.L., and Harrison, D.G. (2002). Detection of superoxide in vascular tissue. *Arterioscler Thromb Vasc Biol* 22, 1761-1768.
- Murrant, C.L., and Reid, M.B. (2001). Detection of reactive oxygen and reactive nitrogen species in skeletal muscle. *Microsc Res Tech* 55, 236-248.
- Mysara, M., Leys, N., Raes, J., and Monsieurs, P. (2016). IPED: a highly efficient denoising tool for Illumina MiSeq Paired-end 16S rRNA gene amplicon sequencing data. *BMC bioinformatics* 17, 192.
- Mysara, M., Njima, M., Leys, N., Raes, J., and Monsieurs, P. (2017). From reads to operational taxonomic units: an ensemble processing pipeline for MiSeq amplicon sequencing data. *GigaScience* 6, 1-10.
- Mysara, M., Saey, Y., Leys, N., Raes, J., and Monsieurs, P. (2015). CATCh, an ensemble classifier for chimera detection in 16S rRNA sequencing studies. *Applied and environmental microbiology* 81, 1573-1584.
- Najdegerami, E.H., Tran, T.N., Defoirdt, T., Marzorati, M., Sorgeloos, P., Boon, N., and Bossier, P. (2012). Effects of poly-β-hydroxybutyrate (PHB) on Siberian sturgeon (*Acipenser baeri*) fingerlings performance and its gastrointestinal tract microbial community. *FEMS microbiology ecology* 79, 25-33.
- Narváez-Zapata, J., Tebbe, C., and Ortega-Morales, B. (2005). Molecular diversity and biomass of epilithic biofilms from intertidal rocky shores in the Gulf of Mexico. *Biofilms* 2, 93-103.
- Navarre-Sitchler, A., and Brantley, S. (2007). Basalt weathering across scales. *Earth and Planetary Science Letters* 261, 321-334.
- Neal, C.R., and Taylor, L.A. (1992). Petrogenesis of mare basalts: A record of lunar volcanism. *Geochimica et Cosmochimica Acta* 56, 2177-2211.

- Nebe-Von-Caron, G., and Mueller, S. (2007). Bugs in the Beam. ed) *JP Robinson, MMK Holdings, Cytometry* 10.
- Nebe-Von-Caron, G., Stephens, P., Hewitt, C., Powell, J., and Badley, R. (2000). Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *J Microbiol Methods* 42, 97-114.
- Neidhardt, F.C., Ingraham, J.L., and Schaechter, M. (1990). Physiology of the bacterial cell: a molecular approach.
- Nelson, M., Pechurkin, N.S., Allen, J.P., Somova, L.A., and Gitelson, J.I. (2010). "Closed ecological systems, space life support and biospherics," in *Environmental Biotechnology*. Springer), 517-565.
- Nemergut, D.R., Anderson, S.P., Cleveland, C.C., Martin, A.P., Miller, A.E., Seimon, A., and Schmidt, S.K. (2007). Microbial community succession in an unvegetated, recently deglaciated soil. *Microbial ecology* 53, 110-122.
- Nesbitt, H., and Wilson, R. (1992). Recent chemical weathering of basalts. *American Journal of Science* 292, 740-777.
- Neu, T.R., Kuhllicke, U., and Lawrence, J.R. (2002). Assessment of fluorochromes for two-photon laser scanning microscopy of biofilms. *Applied and environmental microbiology* 68, 901-909.
- Nevin, K.P., and Lovley, D.R. (2002). Mechanisms for Fe (III) oxide reduction in sedimentary environments. *Geomicrobiology Journal* 19, 141-159.
- Newman, D.K. (2010). Feasting on minerals. *Science* 327, 793-794.
- Newman, D.K., and Banfield, J.F. (2002). Geomicrobiology: how molecular-scale interactions underpin biogeochemical systems. *Science* 296, 1071-1077.
- Ng, D.H., Kumar, A., and Cao, B. (2016). Microorganisms meet solid minerals: interactions and biotechnological applications. *Applied microbiology and biotechnology* 100, 6935-6946.
- Nickerson, C.A., Ott, C.M., Wilson, J.W., Ramamurthy, R., and Pierson, D.L. (2004). Microbial responses to microgravity and other low-shear environments. *Microbiology and Molecular Biology Reviews* 68, 345-361.
- Nielsen, T.H., Sjøholm, O.R., and Sørensen, J. (2009). Multiple physiological states of a *Pseudomonas fluorescens* DR54 biocontrol inoculant monitored by a new flow cytometry protocol. *FEMS Microbiol Ecol* 67, 479-490.
- Nies, D.H. (2016). The biological chemistry of the transition metal "transportome" of *Cupriavidus metallidurans*. *Metallomics* 8, 481-507.
- Northup, D., Melim, L., Spilde, M., Hathaway, J., Garcia, M., Moya, M., Stone, F., Boston, P., Dapkevicius, M., and Riquelme, C. (2011). Lava cave microbial communities within mats and secondary mineral deposits: implications for life detection on other planets. *Astrobiology* 11, 601-618.
- Nowroth, V., Marquart, L., and Jendrossek, D. (2016). Low temperature-induced viable but not culturable state of *Ralstonia eutropha* and its relationship to accumulated polyhydroxybutyrate. *FEMS Microbiology Letters* 363, fnw249.
- Ntarlagiannis, D., Atekwana, E.A., Hill, E.A., and Gorby, Y. (2007). Microbial nanowires: Is the subsurface "hardwired"? *Geophysical Research Letters* 34.
- Nüsslein, K., and Tiedje, J.M. (1998). Characterization of the dominant and rare members of a young Hawaiian soil bacterial community with small-subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. *Applied and environmental microbiology* 64, 1283-1289.
- Oelkers, E.H., and Gislason, S.R. (2001). The mechanism, rates and consequences of basaltic glass dissolution: I. An experimental study of the dissolution rates of basaltic glass as a function of aqueous Al, Si and oxalic acid concentration at 25 C and pH= 3 and 11. *Geochimica et Cosmochimica Acta* 65, 3671-3681.
- Oelkers, E.H., and Schott, J. (1998). Does organic acid adsorption affect alkali-feldspar dissolution rates? *Chemical Geology* 151, 235-245.
- Oelkers, E.H., and Schott, J. (2001). An experimental study of enstatite dissolution rates as a function of pH, temperature, and aqueous Mg and Si concentration, and the

- mechanism of pyroxene/pyroxenoid dissolution. *Geochimica et Cosmochimica Acta* 65, 1219-1231.
- Oh, T.-J., Han, S.-R., Kang, S., Park, H., and Kim, A.Y. (2016). Complete genome sequence of the xylan-degrading *Mucilaginibacter* sp. strain PAMC26640 isolated from an Arctic lichen. *Journal of biotechnology* 227, 23-24.
- Okibe, N., and Johnson, D.B. (2004). Biooxidation of pyrite by defined mixed cultures of moderately thermophilic acidophiles in pH-controlled bioreactors: Significance of microbial interactions. *Biotechnology and bioengineering* 87, 574-583.
- Olcott, A.N., Sessions, A.L., Corsetti, F.A., Kaufman, A.J., and De Oliveira, T.F. (2005). Biomarker evidence for photosynthesis during Neoproterozoic glaciation. *Science* 310, 471-474.
- Oliver, J.D. (2005). The viable but nonculturable state in bacteria. *J Microbiol* 43, 93-100.
- Oliver, J.D. (2016). The viable but nonculturable state for bacteria: status update.
- Olsson-Francis, K., and Cockell, C.S. (2010a). Experimental methods for studying microbial survival in extraterrestrial environments. *Journal of Microbiological Methods* 80, 1-13.
- Olsson-Francis, K., and Cockell, C.S. (2010b). Use of cyanobacteria for in-situ resource use in space applications. *Planetary and Space Science* 58, 1279-1285.
- Olsson-Francis, K., De La Torre, R., and Cockell, C.S. (2010a). Isolation of novel extreme-tolerant cyanobacteria from a rock-dwelling microbial community by using exposure to low Earth orbit. *Applied and environmental microbiology* 76, 2115-2121.
- Olsson-Francis, K., Pearson, V., Boardman, C., Schofield, P., Oliver, A., and Summers, S. (2015). A culture-independent and culture-dependent study of the bacteria community from the bedrock soil interface. *Advances in Microbiology* 5, 842-857.
- Olsson-Francis, K., R, V.a.N.H., Mergeay, M., Leys, N., and Cockell, C.S. (2010b). Microarray analysis of a microbe-mineral interaction. *Geobiology* 8, 446-456.
- Olsson-Francis, K., Simpson, A., Wolff-Boenisch, D., and Cockell, C. (2012). The effect of rock composition on cyanobacterial weathering of crystalline basalt and rhyolite. *Geobiology* 10, 434-444.
- Omelson, C.R. (2008). Endolithic microbial communities in polar desert habitats. *Geomicrobiology Journal* 25, 404-414.
- Onofri, S., De La Torre, R., De Vera, J.-P., Ott, S., Zucconi, L., Selbmann, L., Scalzi, G., Venkateswaran, K.J., Rabbow, E., and Sánchez Iñigo, F.J. (2012). Survival of rock-colonizing organisms after 1.5 years in outer space. *Astrobiology* 12, 508-516.
- Orofino, V., Blanco, A., D'elia, M., Licchelli, D., Fonti, S., and Marzo, G. (2010). Study of terrestrial fossils in phyllosilicate-rich soils: Implication in the search for biosignatures on Mars. *Icarus* 208, 202-206.
- Óskarsson, H., Arnalds, Ó., Gudmundsson, J., and Gudbergsson, G. (2004). Organic carbon in Icelandic Andosols: geographical variation and impact of erosion. *Catena* 56, 225-238.
- Panda, S., Mishra, S., and Akcil, A. (2016). Bioremediation of acidic mine effluents and the role of sulfidogenic biosystems: a mini-review. *Euro-Mediterranean Journal for Environmental Integration* 1, 8.
- Papadimitriou, K., Pratsinis, H., Nebe-Von-Caron, G., Kletsas, D., and Tsakalidou, E. (2007). Acid tolerance of *Streptococcus macedonicus* as assessed by flow cytometry and single-cell sorting. *Appl Environ Microbiol* 73, 465-476.
- Papida, S., Murphy, W., and May, E. (2000). Enhancement of physical weathering of building stones by microbial populations. *International biodeterioration & biodegradation* 46, 305-317.
- Papike, J., Simon, S., and Laul, J. (1982). The lunar regolith: Chemistry, mineralogy, and petrology. *Reviews of Geophysics* 20, 761-826.
- Papike, J.J. (1998). Comparative planetary mineralogy; chemistry of melt-derived pyroxene, feldspar, and olivine. *Reviews in Mineralogy and Geochemistry* 36, 7.1-7.11.
- Park, J.M., Kim, T.Y., and Lee, S.Y. (2011). Genome-scale reconstruction and in silico analysis of the *Ralstonia eutropha* H16 for polyhydroxyalkanoate synthesis,

- lithoautotrophic growth, and 2-methyl citric acid production. *BMC systems biology* 5, 101.
- Paul, D., Kumbhare, S.V., Mhatre, S.S., Chowdhury, S.P., Shetty, S.A., Marathe, N.P., Bhute, S., and Shouche, Y.S. (2015). Exploration of microbial diversity and community structure of lonar lake: the only hypersaline meteorite crater lake within basalt rock. *Frontiers in microbiology* 6.
- Pedrós-Alió, C., Mas-Castellà, J., Mas, J., and Guerrero, R. (1990). "Polyhydroxyalkanoate accumulation in planktonic and anaerobic environments," in *Novel Biodegradable Microbial Polymers*. Springer), 263-274.
- Peeters, E., Nelis, H.J., and Coenye, T. (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of microbiological methods* 72, 157-165.
- Pizarro, H., Izaguirre, I., and Tell, G. (1996). Epilithic algae from a freshwater stream at Hope Bay, Antarctica. *Antarctic Science* 8, 161-167.
- Pokrovsky, O., Shirokova, L., Bénézech, P., Schott, J., and Golubev, S. (2009). Effect of organic ligands and heterotrophic bacteria on wollastonite dissolution kinetics. *American Journal of Science* 309, 731-772.
- Polz, M.F., and Cavanaugh, C.M. (1998). Bias in template-to-product ratios in multitemplate PCR. *Applied and environmental Microbiology* 64, 3724-3730.
- Popa, R., Smith, A.R., Popa, R., Boone, J., and Fisk, M. (2012). Olivine-respiring bacteria isolated from the rock-ice interface in a lava-tube cave, a Mars analog environment. *Astrobiology* 12, 9-18.
- Porter, J., Diaper, J., Edwards, C., and Pickup, R. (1995). Direct measurements of natural planktonic bacterial community viability by flow cytometry. *Appl Environ Microbiol* 61, 2783-2786.
- Povolo, S., and Casella, S. (2000). A critical role for aniA in energy-carbon flux and symbiotic nitrogen fixation in *Sinorhizobium meliloti*. *Archives of microbiology* 174, 42-49.
- Prakash, B., Veeregowda, B., and Krishnappa, G. (2003). Biofilms: a survival strategy of bacteria. *Current science* 85, 1299-1307.
- Prescott, L., Harley, J., and Klein, D. (2004). Microbiology, sixth. *The McGraw-Hill Companies Inc* 526, 937-961.
- Prest, E.I., Hammes, F., Kotzsch, S., Van Loosdrecht, M.C., and Vrouwenvelder, J.S. (2013). Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method. *Water Res* 47, 7131-7142.
- Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glöckner, F.O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic acids research* 35, 7188-7196.
- Pyle, B.H., Broadaway, S.C., and Mcfeters, G.A. (1995). A rapid, direct method for enumerating respiring enterohemorrhagic *Escherichia coli* O157: H7 in water. *Applied and environmental microbiology* 61, 2614-2619.
- Quesada, A., Vincent, W.F., and Lean, D.R. (1999). Community and pigment structure of Arctic cyanobacterial assemblages: the occurrence and distribution of UV-absorbing compounds. *FEMS Microbiology Ecology* 28, 315-323.
- Quintana, N., Van Der Kooy, F., Van De Rhee, M.D., Voshol, G.P., and Verpoorte, R. (2011). Renewable energy from Cyanobacteria: energy production optimization by metabolic pathway engineering. *Applied microbiology and biotechnology* 91, 471-490.
- Rabbow, E., Rettberg, P., Barczyk, S., Bohmeier, M., Parpart, A., Panitz, C., Horneck, G., Von Heise-Rotenburg, R., Hoppenbrouwers, T., and Willnecker, R. (2012). EXPOSE-E: an ESA astrobiology mission 1.5 years in space. *Astrobiology* 12, 374-386.
- Raberg, M., Voigt, B., Hecker, M., and Steinbüchel, A. (2014). A closer look on the polyhydroxybutyrate-(PHB-) negative phenotype of *Ralstonia eutropha* PHB-4. *PLoS one* 9, e95907.
- Radajewski, S., Ineson, P., Parekh, N.R., and Murrell, J.C. (2000). Stable-isotope probing as a tool in microbial ecology. *Nature* 403, 646-649.

- Raksajit, W., Satchasataporn, K., Lehto, K., Mäenpää, P., and Incharoensakdi, A. (2012). Enhancement of hydrogen production by the filamentous non-heterocystous cyanobacterium *Arthrospira* sp. PCC 8005. *international journal of hydrogen energy* 37, 18791-18797.
- Ramette, A. (2007). Multivariate analyses in microbial ecology. *FEMS microbiology ecology* 62, 142-160.
- Rault, A., Bouix, M., and Beal, C. (2008). Dynamic analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 physiological characteristics during fermentation. *Appl Microbiol Biotechnol* 81, 559-570.
- Rault, A., Bouix, M., and Beal, C. (2009). Fermentation pH influences the physiological-state dynamics of *Lactobacillus bulgaricus* CFL1 during pH-controlled culture. *Appl Environ Microbiol* 75, 4374-4381.
- Rawlings, D.E. (2005). Characteristics and adaptability of iron-and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. *Microbial cell factories* 4, 13.
- Rawlings, D.E., and Johnson, D.B. (2007). The microbiology of biomining: development and optimization of mineral-oxidizing microbial consortia. *Microbiology* 153, 315-324.
- Reasoner, D., and Geldreich, E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* 49, 1-7.
- Reguera, G., Nevin, K.P., Nicoll, J.S., Covalla, S.F., Woodard, T.L., and Lovley, D.R. (2006). Biofilm and nanowire production leads to increased current in *Geobacter sulfurreducens* fuel cells. *Applied and environmental microbiology* 72, 7345-7348.
- Reith, F., Etschmann, B., Grosse, C., Moors, H., Benotmane, M.A., Monsieurs, P., Grass, G., Doonan, C., Vogt, S., and Lai, B. (2009). Mechanisms of gold biomineralization in the bacterium *Cupriavidus metallidurans*. *Proceedings of the National Academy of Sciences* 106, 17757-17762.
- Rettberg, P., Anesio, A.M., Baker, V.R., Baross, J.A., Cady, S.L., Detsis, E., Foreman, C.M., Hauber, E., Ori, G.G., and Pearce, D.A. (2016). "Planetary protection and mars special regions—a suggestion for updating the definition". Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA).
- Rezaeinejad, S., and Ivanov, V. (2011). Heterogeneity of *Escherichia coli* population by respiratory activity and membrane potential of cells during growth and long-term starvation. *Microbiol Res* 166, 129-135.
- Richardson, A.E. (2001). Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Functional Plant Biology* 28, 897-906.
- Rieseberg, M., Kasper, C., Reardon, K.F., and Scheper, T. (2001). Flow cytometry in biotechnology. *Appl Microbiol Biotechnol* 56, 350-360.
- Ro, D.-K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., and Kirby, J. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940-943.
- Rogers, J.R., and Bennett, P.C. (2004). Mineral stimulation of subsurface microorganisms: release of limiting nutrients from silicates. *Chemical Geology* 203, 91-108.
- Rojas, L.A., Yáñez, C., González, M., Lobos, S., Smalla, K., and Seeger, M. (2011). Characterization of the metabolically modified heavy metal-resistant *Cupriavidus metallidurans* strain MSR33 generated for mercury bioremediation. *PloS one* 6, e17555.
- Rosenzweig, J.A., Ahmed, S., Eunson, J., and Chopra, A.K. (2014). Low-shear force associated with modeled microgravity and spaceflight does not similarly impact the virulence of notable bacterial pathogens. *Applied microbiology and biotechnology* 98, 8797-8807.
- Roszak, D., and Colwell, R. (1987). Survival strategies of bacteria in the natural environment. *Microbiol Rev* 51, 365.
- Ruzicka, A., Snyder, G.A., and Taylor, L.A. (2001). Comparative geochemistry of basalts from the Moon, Earth, HED asteroid, and Mars: Implications for the origin of the Moon. *Geochimica et Cosmochimica Acta* 65, 979-997.

- Ryder, G., and Schuraytz, B.C. (2001). Chemical variation of the large Apollo 15 olivine-normative mare basalt rock samples. *Journal of Geophysical Research: Planets* 106, 1435-1451.
- Sancho, L.G., De La Torre, R., Horneck, G., Ascaso, C., De Los Rios, A., Pintado, A., Wierzbos, J., and Schuster, M. (2007). Lichens survive in space: results from the 2005 LICHENS experiment. *Astrobiology* 7, 443-454.
- Sand, W., Gehrke, T., Jozsa, P.-G., and Schippers, A. (2001). (Bio) chemistry of bacterial leaching—direct vs. indirect bioleaching. *Hydrometallurgy* 59, 159-175.
- Santelli, C.M., Edgcomb, V.P., Bach, W., and Edwards, K.J. (2009). The diversity and abundance of bacteria inhabiting seafloor lavas positively correlate with rock alteration. *Environmental Microbiology* 11, 86-98.
- Sato, Y., Nishihara, H., Yoshida, M., Watanabe, M., Rondal, J.D., Concepcion, R.N., and Ohta, H. (2006). *Cupriavidus pinatubonensis* sp. nov. and *Cupriavidus laharis* sp. nov., novel hydrogen-oxidizing, facultatively chemolithotrophic bacteria isolated from volcanic mudflow deposits from Mt. Pinatubo in the Philippines. *International journal of systematic and evolutionary microbiology* 56, 973-978.
- Sato, Y., Nishihara, H., Yoshida, M., Watanabe, M., Rondal, J.D., and Ohta, H. (2004). Occurrence of hydrogen-oxidizing *Ralstonia* species as primary microorganisms in the Mt. Pinatubo volcanic mudflow deposits. *Soil Science and Plant Nutrition* 50, 855-861.
- Schloss, P.D., and Handelsman, J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and environmental microbiology* 71, 1501-1506.
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., and Robinson, C.J. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology* 75, 7537-7541.
- Schultz, P.H., and Wrobel, K.E. (2012). The oblique impact Hale and its consequences on Mars. *Journal of Geophysical Research: Planets* 117.
- Schulz, S., Brankatschk, R., Dümig, A., Kögel-Knabner, I., Schlöter, M., and Zeyer, J. (2013). The role of microorganisms at different stages of ecosystem development for soil formation. *Biogeosciences* 10, 3983-3996.
- Schwandt, C., Hamilton, J.A., Fray, D.J., and Crawford, I.A. (2012). The production of oxygen and metal from lunar regolith. *Planetary and Space Science* 74, 49-56.
- Seiffert, F., Bandow, N., Bouchez, J., Von Blanckenburg, F., and Gorbushina, A. (2014). Microbial colonization of bare rocks: laboratory biofilm enhances mineral weathering. *Procedia Earth and Planetary Science* 10, 123-129.
- Seneviratne, G., and Indrasena, I. (2006). Nitrogen fixation in lichens is important for improved rock weathering. *Journal of biosciences* 31, 639-643.
- Shang, L., Jiang, M., and Chang, H.N. (2003). Poly (3-hydroxybutyrate) synthesis in fed-batch culture of *Ralstonia eutropha* with phosphate limitation under different glucose concentrations. *Biotechnology letters* 25, 1415-1419.
- Shapiro, H. (2003). *Practical Flow Cytometry*. United States of America: John Wiley & sons, Inc.
- Shapiro, H.H. (2000). Microbial analysis at the single-cell level: tasks and techniques. *J Microbiol Meth* 42, 3-16.
- Sharma, P.K., Fu, J., Spicer, V., Krokhin, O.V., Cicek, N., Sparling, R., and Levin, D.B. (2016). Global changes in the proteome of *Cupriavidus necator* H16 during poly-(3-hydroxybutyrate) synthesis from various biodiesel by-product substrates. *AMB Express* 6, 36.
- Sharp, Z. (2007). *Principles of stable isotope geochemistry*. Pearson education Upper Saddle River, NJ.

- Shi, L., Günther, S., Hübschmann, T., Wick, L.Y., Harms, H., and Müller, S. (2007). Limits of propidium iodide as a cell viability indicator for environmental bacteria. *Cytometry A* 71, 592-598.
- Silva, F., Lourenco, O., Pina-Vaz, C., Rodrigues, A.G., Queiroz, J.A., and Domingues, F.C. (2010). The Use of DRAQ5 to Monitor Intracellular DNA in *Escherichia coli* by Flow Cytometry. *J Fluoresc* 20, 907-914.
- Singha, K. (2012). A short review on basalt fiber. *International Journal of Textile Science* 1, 19-28.
- Slmb (2012). "Determining the Total Cell Count and Ratios of High and Low Nucleic Acid Content Cells in Freshwater Using Flow Cytometry". (Switzerland: Federal Office of Public Health).
- Smith, A., Popa, R., Fisk, M., Nielsen, M., Wheat, C.G., Jannasch, H.W., Fisher, A.T., Becker, K., Sievert, S.M., and Flores, G. (2011). In situ enrichment of ocean crust microbes on igneous minerals and glasses using an osmotic flow-through device. *Geochemistry, Geophysics, Geosystems* 12.
- Staudigel, H., Furnes, H., Mcloughlin, N., Banerjee, N.R., Connell, L.B., and Templeton, A. (2008). 3.5 billion years of glass bioalteration: Volcanic rocks as a basis for microbial life? *Earth-Science Reviews* 89, 156-176.
- Staudigel, H., and Hart, S.R. (1983). Alteration of basaltic glass: Mechanisms and significance for the oceanic crust-seawater budget. *Geochimica et Cosmochimica Acta* 47, 337-350.
- Staudigel, H., Yayanos, A., Chastain, R., Davies, G., Verdurmen, E.T., Schiffman, P., Bourcier, R., and De Baar, H. (1998). Biologically mediated dissolution of volcanic glass in seawater. *Earth and Planetary Science Letters* 164, 233-244.
- Stepanović, S., Ćirković, I., and Ranin, L. (2004). Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Letters in applied microbiology* 38, 428-432.
- Stewart, E.J. (2012). Growing unculturable bacteria. *J. Bacteriol* 194, 4151-4160.
- Stewart, P.S., and Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. *The lancet* 358, 135-138.
- Stockmann, G.J., Wolff-Boenisch, D., Gislason, S.R., and Oelkers, E.H. (2011). Do carbonate precipitates affect dissolution kinetics? 1: Basaltic glass. *Chemical Geology* 284, 306-316.
- Stolz, J.F., and Oremland, R.S. (2011). *Microbial metal and metalloid metabolism: advances and applications*. American Society for Microbiology Press.
- Stoodley, P., Sauer, K., Davies, D., and Costerton, J.W. (2002). Biofilms as complex differentiated communities. *Annual Reviews in Microbiology* 56, 187-209.
- Sträuber, H., and Müller, S. (2010). Viability states of bacteria—specific mechanisms of selected probes. *Cytometry Part A* 77, 623-634.
- Stroncik, N.A., and Schmincke, H.U. (2001). Evolution of palagonite: Crystallization, chemical changes, and element budget. *Geochemistry, Geophysics, Geosystems* 2.
- Stumm, W., Furrer, G., and Kunz, B. (1983). The role of surface coordination in precipitation and dissolution of mineral phases. *Croatica Chemica Acta* 56, 593-611.
- Štyriaková, I., Štyriak, I., and Oberhänsli, H. (2012). Rock weathering by indigenous heterotrophic bacteria of *Bacillus* spp. at different temperature: a laboratory experiment. *Mineralogy and Petrology* 105, 135-144.
- Su, X., Sun, F., Wang, Y., Hashmi, M.Z., Guo, L., Ding, L., and Shen, C. (2015). Identification, characterization and molecular analysis of the viable but nonculturable *Rhodococcus biphenylivorans*. *Scientific reports* 5, 18590.
- Sudek, L.A., Wanger, G., Templeton, A.S., Staudigel, H., and Tebo, B.M. (2017). Submarine Basaltic Glass Colonization by the Heterotrophic Fe (II)-Oxidizing and Siderophore-Producing Deep-Sea Bacterium *Pseudomonas stutzeri* VS-10: The Potential Role of Basalt in Enhancing Growth. *Frontiers in Microbiology* 8.

- Summers, S., Whiteley, A.S., Kelly, L.C., and Cockell, C.S. (2013). Land coverage influences the bacterial community composition in the critical zone of a sub-Arctic basaltic environment. *FEMS microbiology ecology* 86, 381-393.
- Sychev, V.N., Levinskikh, M.A., and Podolsky, I.G. (2008). Biological component of life support systems for a crew in long-duration space expeditions. *Acta Astronautica* 63, 1119-1125.
- Tabak, H.H., Lens, P., Van Hullebusch, E.D., and Dejonghe, W. (2005). Developments in bioremediation of soils and sediments polluted with metals and radionuclides—1. Microbial processes and mechanisms affecting bioremediation of metal contamination and influencing metal toxicity and transport. *Reviews in Environmental Science and Biotechnology* 4, 115-156.
- Tanaka, Y., Yamaguchi, N., and Nasu, M. (2000). Viability of *Escherichia coli* O157: H7 in natural river water determined by the use of flow cytometry. *Journal of applied microbiology* 88, 228-236.
- Tarpey, M.M., Wink, D.A., and Grisham, M.B. (2004). Methods for detection of reactive metabolites of oxygen and nitrogen in vitro and in vivo considerations. *Am J Physiol Regul Integr Comp Physiol* 286, R431–R444.
- Taylor, P.W. (2015). Impact of space flight on bacterial virulence and antibiotic susceptibility. *Infection and drug resistance* 8, 249.
- Taylor, S.R., and McLennan, S. (2009). *Planetary crusts: their composition, origin and evolution*. Cambridge University Press.
- Tebo, B.M., Davis, R.E., Anitori, R.P., Connell, L.B., Schiffman, P., and Staudigel, H. (2015). Microbial communities in dark oligotrophic volcanic ice cave ecosystems of Mt. Erebus, Antarctica. *Frontiers in microbiology* 6, 179.
- Teixeira, L.C., Peixoto, R.S., Cury, J.C., Sul, W.J., Pellizari, V.H., Tiedje, J., and Rosado, A.S. (2010). Bacterial diversity in rhizosphere soil from Antarctic vascular plants of Admiralty Bay, maritime Antarctica. *The ISME journal* 4, 989-1001.
- Templeton, A.S., Staudigel, H., and Tebo, B.M. (2005). Diverse Mn (II)-oxidizing bacteria isolated from submarine basalts at Loihi Seamount. *Geomicrobiology Journal* 22, 127-139.
- Thompson, J., and Ferris, F. (1990). Cyanobacterial precipitation of gypsum, calcite, and magnesite from natural alkaline lake water. *Geology* 18, 995-998.
- Thordarson, T., and Larsen, G. (2007). Volcanism in Iceland in historical time: Volcano types, eruption styles and eruptive history. *Journal of Geodynamics* 43, 118-152.
- Thorseth, I., Furnes, H., and Heldal, M. (1992). The importance of microbiological activity in the alteration of natural basaltic glass. *Geochimica et Cosmochimica Acta* 56, 845-850.
- Thorseth, I., Torsvik, T., Torsvik, V., Daae, F., and Pedersen, R. (2001). Diversity of life in ocean floor basalt. *Earth and Planetary Science Letters* 194, 31-37.
- Tirumalai, M.R., Karouia, F., Tran, Q., Stepanov, V.G., Bruce, R.J., Ott, C.M., Pierson, D.L., and Fox, G.E. (2017). The adaptation of *Escherichia coli* cells grown in simulated microgravity for an extended period is both phenotypic and genomic. *npj Microgravity* 3, 15.
- Tokano, T., and Bish, D.L. (2005). Hydration state and abundance of zeolites on Mars and the water cycle. *Journal of Geophysical Research: Planets* 110.
- Tracy, B.P., Gaida, S.M., and Papoutsakis, E.T. (2010). Flow cytometry for bacteria: enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes. *Curr Opin Biotechnol* 21, 85-99.
- Tremblay, Y.D., Lamarche, D., Chever, P., Haine, D., Messier, S., and Jacques, M. (2013). Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *Journal of dairy science* 96, 234-246.
- Trevors, J. (2011). Viable but non-culturable (VBNC) bacteria: Gene expression in planktonic and biofilm cells. *Journal of microbiological methods* 86, 266-273.

- Tribelli, P.M., and López, N.I. (2011). Poly (3-hydroxybutyrate) influences biofilm formation and motility in the novel Antarctic species *Pseudomonas extremaustralis* under cold conditions. *Extremophiles* 15, 541.
- Tuffin, I.M., Hector, S.B., Deane, S.M., and Rawlings, D.E. (2006). Resistance determinants of a highly arsenic-resistant strain of *Leptospirillum ferriphilum* isolated from a commercial biooxidation tank. *Applied and environmental microbiology* 72, 2247-2253.
- Tyo, K.E., Zhou, H., and Stephanopoulos, G.N. (2006). High-throughput screen for poly-3-hydroxybutyrate in *Escherichia coli* and *Synechocystis* sp strain PCC6803. *Appl Environ Microbiol* 72, 3412-3417.
- Ubal dini, S., Veglio, F., Beolchini, F., Toro, L., and Abbruzzese, C. (2000a). Gold recovery from a refractory pyrrhotite ore by biooxidation. *International journal of mineral processing* 60, 247-262.
- Ubal dini, S., Veglio, F., Fornari, P., and Abbruzzese, C. (2000b). Process flow-sheet for gold and antimony recovery from stibnite. *Hydrometallurgy* 57, 187-199.
- Uroz, S., Calvaruso, C., Turpault, M.-P., and Frey-Klett, P. (2009). Mineral weathering by bacteria: ecology, actors and mechanisms. *Trends in microbiology* 17, 378-387.
- Uroz, S., Kelly, L.C., Turpault, M.-P., Lepleux, C., and Frey-Klett, P. (2015). The mineralosphere concept: mineralogical control of the distribution and function of mineral-associated bacterial communities. *Trends in microbiology* 23, 751-762.
- Valdés, J., Pedroso, I., Quatrini, R., Dodson, R.J., Tettelin, H., Blake, R., Eisen, J.A., and Holmes, D.S. (2008). Acidithiobacillus ferrooxidans metabolism: from genome sequence to industrial applications. *BMC genomics* 9, 597.
- Valenzuela, L., Chi, A., Beard, S., Orell, A., Guilian, N., Shabanowitz, J., Hunt, D.F., and Jerez, C.A. (2006). Genomics, metagenomics and proteomics in biomining microorganisms. *Biotechnology advances* 24, 197-211.
- Van Houdt, R., Monsieurs, P., Mijndonckx, K., Provoost, A., Janssen, A., Mergeay, M., and Leys, N. (2012). Variation in genomic islands contribute to genome plasticity in *Cupriavidus metallidurans*. *BMC genomics* 13, 111.
- Van Nevel, S., Koetzsch, S., Weilenmann, H.-U., Boon, N., and Hammes, F. (2013). Routine bacterial analysis with automated flow cytometry. *Journal of microbiological methods* 94, 73-76.
- Van Overbeek, L.S., Bergervoet, J.H., Jacobs, F.H., and Van Elsas, J.D. (2004). The low-temperature-induced viable-but-nonculturable state affects the virulence of *Ralstonia solanacearum* biovar 2. *Phytopathology* 94, 463-469.
- Vanhavere, F., Genicot, J.L., O'sullivan, D., Zhou, D., Spurný, F., Jadrníčková, I., Sawakuchi, G., and Yuki-hara, E. (2008). DOSimetry of Biological EXperiments in SPace (DOBIES) with luminescence (OSL and TL) and track etch detectors. *Radiation Measurements* 43, 694-697.
- Vaniman, D., Reedy, R., Heiken, G., Olhoeft, G., and Mendell, W. (1991). The lunar environment. *The lunar Sourcebook*, CUP, 27-60.
- Vaughan, D.J., and Lloyd, J.R. (2011). Mineral-organic-microbe interactions: Environmental impacts from molecular to macroscopic scales. *Comptes Rendus Geoscience* 343, 140-159.
- Veal, D., Deere, D., Ferrari, B., Piper, J., and Attfield, P. (2000). Fluorescence staining and flow cytometry for monitoring microbial cells. *Journal of immunological methods* 243, 191-210.
- Vera, M., Krok, B., Bellenberg, S., Sand, W., and Poetsch, A. (2013). Shotgun proteomics study of early biofilm formation process of *Acidithiobacillus ferrooxidans* ATCC 23270 on pyrite. *Proteomics* 13, 1133-1144.
- Verseux, C., Baqué, M., Lehto, K., De Vera, J.-P.P., Rothschild, L.J., and Billi, D. (2016a). Sustainable life support on Mars—the potential roles of cyanobacteria. *International Journal of Astrobiology* 15, 65-92.

- Verseux, C.N., Paulino-Lima, I.G., Baqué, M., Billi, D., and Rothschild, L.J. (2016b). "Synthetic biology for space exploration: promises and societal implications," in *Ambivalences of Creating Life*. Springer), 73-100.
- Vidal-Mas, J., Resina-Pelfort, O., Haba, E., Comas, J., Manresa, A., and Vives-Rego, J. (2001). Rapid flow cytometry - Nile red assessment of PHA cellular content and heterogeneity in cultures of *Pseudomonas aeruginosa* 47T2 (NCIB 40044) grown in waste frying oil. *Antonie Van Leeuwenhoek* 80, 57-63.
- Vilchez-Vargas, R., Geffers, R., Suárez-Diez, M., Conte, I., Waliczek, A., Kaser, V.S., Kralova, M., Junca, H., and Pieper, D.H. (2013). Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system. *Environmental microbiology* 15, 1016-1039.
- Vriezen, J., De Bruijn, F.J., and Nüsslein, K.R. (2012). Desiccation induces viable but non-culturable cells in *Sinorhizobium meliloti* 1021. *AMB express* 2.
- Wacey, D., Kilburn, M.R., Saunders, M., Cliff, J., and Brasier, M.D. (2011). Microfossils of sulphur-metabolizing cells in 3.4-billion-year-old rocks of Western Australia. *Nature Geoscience* 4, 698-702.
- Walford, R.L., Mock, D., Verdery, R., and Maccallum, T. (2002). Calorie restriction in biosphere 2: alterations in physiologic, hematologic, hormonal, and biochemical parameters in humans restricted for a 2-year period. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 57, B211-B224.
- Walsh, J., and Clarke, E. (1982). The role of fractional crystallization in the formation of granitic and intermediate rocks of the Beinn Chaisgidle Centre, Mull, Scotland. *Mineralogical Magazine* 45, 247-255.
- Wang, J., and Bakken, L. (1998). Screening of soil bacteria for poly- β -hydroxybutyric acid production and its role in the survival of starvation. *Microbial ecology* 35, 94-101.
- Wang, Y., Hammes, F., De Roy, K., Verstraete, W., and Boon, N. (2010). Past, present and future applications of flow cytometry in aquatic microbiology. *Trends Biotechnol* 28, 416-424.
- Warren-Rhodes, K.A., Rhodes, K.L., Pointing, S.B., Ewing, S.A., Lacap, D.C., Gómez-Silva, B., Amundson, R., Friedmann, E.I., and McKay, C.P. (2006). Hypolithic cyanobacteria, dry limit of photosynthesis, and microbial ecology in the hyperarid Atacama Desert. *Microbial Ecology* 52, 389-398.
- Warren, P.H., and Wasson, J.T. (1979). The origin of KREEP. *Reviews of Geophysics* 17, 73-88.
- Welch, S., Barker, W., and Banfield, J. (1999). Microbial extracellular polysaccharides and plagioclase dissolution. *Geochimica et Cosmochimica acta* 63, 1405-1419.
- Welch, S., Taunton, A., and Banfield, J. (2002). Effect of microorganisms and microbial metabolites on apatite dissolution. *Geomicrobiology Journal* 19, 343-367.
- Welch, S.A., and Banfield, J.F. (2002). Modification of olivine surface morphology and reactivity by microbial activity during chemical weathering. *Geochimica et Cosmochimica Acta* 66, 213-221.
- Welch, S.A., and Ullman, W.J. (1993). The effect of organic acids on plagioclase dissolution rates and stoichiometry. *Geochimica et Cosmochimica Acta* 57, 2725-2736.
- Werner, S.C. (2009). The global martian volcanic evolutionary history. *Icarus* 201, 44-68.
- Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998). Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences* 95, 6578-6583.
- Wilson, J.W., Ott, C.M., Quick, L., Davis, R., Zu Bentrup, K.H., Crabbé, A., Richter, E., Sarker, S., Barrila, J., and Porwollik, S. (2008). Media ion composition controls regulatory and virulence response of Salmonella in spaceflight. *PLoS One* 3, e3923.
- Wilson, L. (2009). Volcanism in the solar system. *Nature Geoscience* 2, 389-397.
- Wolf, R., Woodrow, A., and Anders, E. (Year). "Lunar basalts and pristine highland rocks- Comparison of siderophile and volatile elements", in: *Lunar and Planetary Science Conference Proceedings*, 2107-2130.

- Wolff-Boenisch, D., Gislason, S.R., Oelkers, E.H., and Putnis, C.V. (2004). The dissolution rates of natural glasses as a function of their composition at pH 4 and 10.6, and temperatures from 25 to 74 C. *Geochimica et Cosmochimica Acta* 68, 4843-4858.
- Wu, L., Jacobson, A.D., Chen, H.-C., and Hausner, M. (2007). Characterization of elemental release during microbe–basalt interactions at T= 28 C. *Geochimica et Cosmochimica Acta* 71, 2224-2239.
- Wyatt, M.B., and Mcsween, H.Y. (2002). Spectral evidence for weathered basalt as an alternative to andesite in the northern lowlands of Mars. *Nature* 417, 263-266.
- Wynn-Williams, D., Edwards, H., Newton, E., and Holder, J. (2002). Pigmentation as a survival strategy for ancient and modern photosynthetic microbes under high ultraviolet stress on planetary surfaces. *International journal of Astrobiology* 1, 39-49.
- Yamaguchi, N., and Nasu, M. (1997). Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *Journal of Applied Microbiology* 83, 43-52.
- Yue, J.C., and Clayton, M.K. (2005). A similarity measure based on species proportions. *Communications in Statistics-Theory and Methods* 34, 2123-2131.
- Zea, L., Prasad, N., Levy, S.E., Stodieck, L., Jones, A., Shrestha, S., and Klaus, D. (2016). A Molecular Genetic Basis Explaining Altered Bacterial Behavior in Space. *PloS one* 11, e0164359.
- Zhi, W., Ge, Z., He, Z., and Zhang, H. (2014). Methods for understanding microbial community structures and functions in microbial fuel cells: a review. *Bioresource technology* 171, 461-468.
- Zipper, H., Brunner, H., Bernhagen, J., and Vitzthum, F. (2004). Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res* 32, e103.

Appendix

Appendix 1: Basalt compositions of all the basalt tested within this work

wt (%)	SCK1	SCK2	SCK3	SCK4	SCK5	SCK6	SCK7
SiO ₂ [°]	49.41	49.15	49.42	51.1	51.51	47.41	48.78
Al ₂ O ₃ [°]	13.18	13.24	13.38	15.16	13.45	13.85	17.28
FeO ^{°°}	12.3	9,00	11.1	9.53	8.2	7.3	7.3
Fe ₂ O ₃ [°]	1.81	5.05	1.84	nd	2.21	2.58	1.07
MnO [°]	0.23	0.23	0.22	0.18	0.16	0.17	0.15
MgO [°]	5.64	5.76	5.88	8.87	7.16	9.5	7.61
CaO [°]	9.96	9.5	9.97	12.36	10.81	12.39	12.32
Na ₂ O [°]	2.33	2.3	2.34	1.76	2.34	2.12	2.4
K ₂ O [°]	0.36	0.32	0.33	0.05	0.35	0.63	0.17
TiO ₂ [°]	2,00	1.99	1.84	0.91	1.91	1.51	1.22
P ₂ O ₅ [°]	0.23	0.22	0.22	0.08	0.21	0.28	0.14
LOI	0.07	2.19	1.47	0.47	0.37	1.41	0.18

°SD of 0.01%

°°SD of 0.1%

nd: not determined

ppm*	SCK1	SCK2	SCK3	SCK4	SCK5	SCK6	SCK7
V	438	420	405	301.4	278	264	238
Sr	151	147	148	64	282	339	208
Zn	130	130	120	79.4	100	100	70
Cu	120	160	130	95.3	140	80	170
Cr	120	120	140	351.1	290	300	330
Zr	118	120	119	46.8	114	104	66
Ba	89	90	89	2.5	75	225	50
Ni	80	80	90	131.6	120	180	160
Sc	44	44	43	47.6	30	34	32
Y	30	30	29	25.1	20	20	17
Nb	11	11	11	0.7	7	30	7
Rb	9	7	7	0.5	5	17	2

*SD of 0.1

Appendix 2: Material and methods section Chapter 6

Preparation of bacteria cultures

Two bacterial strains were used. The Gram-positive *Lactobacillus brevis* LMG 18022 and the Gram-negative *Cupriavidus metallidurans* CH34. Both were grown overnight at 28°C in aerobic conditions in liquid media, MRS broth (Oxoid, UK) and LB broth (Oxoid, UK), respectively. As example of a mixed microbial culture, bottled Evian water was used. Prior flow cytometry analysis bacterial cultures were diluted in phosphate buffered saline (PBS) solution to a concentration around 10⁵ cells/mL prior analysis with the flow cytometer in order to guarantee a good detection of the particles.

Flow cytometry and used stains

Bacteria were analyzed on a Accuri C6 (BD, Erembodegem) with a blue (488 nm) and red (640 nm) laser. Standard optical filters were used and included FL-1 (530/30 nm), FL-2 (585/40 nm) and FL-3 (670 LP) for the blue laser and FL-4 (675/25 nm) for the red laser. The tested dyes include DiBAC₄(3) (Sigma Aldrich, U.S.A), cFDA (Sigma Aldrich, U.S.A) and cFDA-SE (Sigma Aldrich, U.S.A) and were detected with FL-1. The dyes dihydroethidium (Sigma Aldrich, U.S.A) and Nile red (Sigma Aldrich, U.S.A) were detected with FL-3 and the red excitable SYTO dyes 17, 59-64 (Thermo Fisher, U.S.A.) were detected with FL-4.

Assessment of staining kinetics

Staining kinetics were assessed by staining 1.5 mL of a bacterial suspension (as described above) and measuring continuously for 40 min (or one hour for HE). The sample was incubated at 37°C during the measurement period.

Assessment of optimal conditions for the single stains

For all SYTO stains the concentration, incubation time and the effect of the stain enhancer EDTA were tested for both bacteria. The final dye concentrations were 5µM, 0.5µM and 0.05µM and the incubation times were 0 min, 15 min and 30 min. The final concentration of EDTA was 5mM. For all vital stains one single concentration was used based on literature review, titration and the optimal stain concentration was chosen based on maximized signal to background distinction. The final concentrations chosen were 5µM, 1µM, 0.2µM, 10µM and 0.3µM for dihydroethidium, DiBAC₄(3), cFDA-SE, cFDA and Nile Red respectively. For all SYTO stains a sample filtered over a 0.22µm filter (Merck Millipore, U.S.A.) was used as a negative control. For DiBAC₄(3), cFDA and cFDA-SE a sample was heat killed by incubation for 30 min at 100°C and used as

a negative control. An oxidized sample was prepared by adding 10µl of 30% H₂O₂ (Sigma Aldrich, U.S.A.) to 500 µl of sample. After 30 min of incubation the suspension was centrifuged and washed 3 times with filtered PBS to remove the H₂O₂ as to avoid bleaching of the stains. This sample was used as positive control for dihydroethidium staining.

Assessment of triple staining

Following triple stains were tested DiBAC₄(3)/ dihydroethidium/ SYTO 60, DiBAC₄(3)/ Nile red/ SYTO 60, cFDA/ dihydroethidium/ SYTO 60, cFDA/ Nile Red/ SYTO 60, cFDA-SE/ dihydroethidium/ SYTO 60, cFDA-SE/ Nile Red/ SYTO 60. For each triple stain, all double stain combinations were tested and all single stains as well. The stains were tested on both bacteria and on all controls described previously. Finally a mixture of different samples was made in order to have a positive and negative signal for each stain. All samples were incubated at 37°C for 30 min. After flow cytometry read out, a gating protocol was established. For the double stains, First both FL1 positive and FL3 positive cells were gated. Both positive and negative FL1 and FL3 populations were then gated in the opposite channel (e.g. the FL3 positive population was gated in the FL1 channel). This made it possible to distinguish the FL1+,FL3+, FL1-,FL3-, FL1+/FL3+, FL1-/FL3+, FL1+/FL3-, FL1-/FL3- populations. After establishing the right thresholds and gates this could be repeated for the triple stain as samples were now also visualized in the FL4 channel distinguishing cells from background, and establishing the same gates as for the double stains.

Stability

Mixed population samples of either *L. brevis* LMG 18022 or *C. metallidurans* CH34 were prepared and stained with any of the triple combinations mentioned in the previous section, subsamples of this stained batch were then loaded in a 96 well plate and measured sequentially with the flow cytometer after 30 min of incubation at 37°C. Cell counts were then determined by applying the gating protocol mentioned in the previous section allowing to look at the stability of these counts over the time needed to process the complete 96-well plate (about 1h30 min).

Appendix

Abstract

Microorganisms interact with minerals for sustaining their survival and growth. Bioweathering of crustal rocks is one of the principal processes controlling the geochemical cycling of elements at the Earth's surface and basalt weathering acts as a major atmospheric CO₂ sink. Next to their involvement in bioweathering and the geochemical cycle, microbe-mineral interactions have already proved their relevance in agricultural and industrial applications such as enhancing soil fertility, bioremediation and bioremediation of inorganic pollutants and biomining. More recently, microbe-mineral interactions have also become of interest for space exploration missions. Supplies generated from endogenous material on planets and asteroids, so called “in situ material” such as the regolith and rocks, could reduce the costs and dependence for supplies from Earth.

To increase the knowledge about microbe-mineral interactions in space and the potential for in situ resource utilization (ISRU), and in preparation of the ESA BioRock space flight experiment, the interaction of the bacterium *Cupriavidus metallidurans* CH34 with basalt, a volcanic moon-analog rock, was investigated. The bacterial presence on basalt and its impact on weathering, as well as the effect of basalt composition on element leaching was studied. In addition, survival and the potential impact of space environmental conditions such as microgravity on these interactions were determined.

First, the different communities present on a chronosequence of basalt deposits were determined as well as the relationship between the bacterial presence, composition and age of the lava basalt. The young and intermediate flows appeared to be dominated by *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*, which could be the first to colonize new terrestrial basalt formations through their autotrophic, photoheterotrophic and chemolithotrophic abilities, while in the old flow *Firmicutes* and *Proteobacteria* appeared to dominate. Our results could indicate that especially N₂-fixers such as *Planctomycetaceae*, *Rhizobiales* *Sphingobacteriales*, *Acetobacteraceae*, *Clostridium*, *Chitinophagaceae*, and heterotrophs such as *Ralstonia*, were responsible for shift of the population to the old flow, allowing further

rhizosphere and soil bacteria to colonize this environment and contribute to habitability development.

Next, the impact of different basalt compositions on nutrient leaching and subsequent support of growth of the bacterium *Cupriavidus metallidurans* CH34 was investigated. Seven different basalts were examined, each leaching different amounts of calcium, iron, magnesium and phosphorus. In iron limiting media *C. metallidurans* growth could be restored by all basalt tested, while only two basalts could restore the growth in magnesium-limiting medium impacting both growth rate as well as final density reached. Lack of phosphorus could not be complemented by any of the basalts tested showing that different basalt compositions release different amount of nutrients, not related to basalt composition, impacting bacterial growth.

In addition, long-term starvation of *C. metallidurans* in mineral water and in the presence of basalt was studied, probing the potential of ISRU. Our results indicated that basalt had a positive effect on survival through the release of elements such as sodium, potassium, and phosphate in the water, lessening some of the detrimental effects of starvation. Thereby, cells became more culturable, contained more ATP and accumulated less PHB in the presence of basalt. Cells will also start forming a biofilm on basalt, to protect them from the harsher planktonic environment. Space flight conditions had a positive effect on survival and cells were more culturable, contained more ATP and more cells were present which were intact and active while fewer cells lost their membrane potential while less biofilm is formed. Element analysis showed that cells in the presence of basalt during the space flight conditions release copper while also taking up phosphate.

Finally, in support of our work, multicolor flow cytometry was validated as a good approach to estimate and assess multiple features at once and to characterize in detail bacterial physiology and the heterogeneity of a community. We demonstrated that red-excitable SYTO dyes could be used as counterstain in combination with other functional dyes. Our results showed that the efficiency can differ between the tested organisms, indicating that optimization is necessary for accurate functional measurements. Furthermore, it was shown that dye combination influenced the results as well as stability and that appropriate controls and compensation are crucial for a correct analysis.

Samenvatting

Micro-organismen kunnen inwerken op gesteenten om mineralen vrij te zetten, en zo hun overleving en groei te bevorderen. Deze interacties spelen ook een rol bij verweringsprocessen en dragen zo bij aan de geo-chemische cycli van verschillende elementen. Daarnaast kunnen deze interacties ook toegepast worden in verschillende industriële processen zoals “biomining”, waarbij belangrijke elementen zoals ijzer en koper kunnen gewonnen worden uit ertsen, en kunnen ze gebruikt worden om bodemvruchtbaarheid te bevorderen. Deze interacties zouden ook toegepast kunnen worden, in de toekomst, voor lange ruimtevaart missies. Aangezien het technisch en economisch niet haalbaar is om alle middelen, nodig voor deze lange ruimtevaart missies, vanuit de Aarde mee te nemen, is onderzoek nu gericht om de haalbaarheid te bestuderen van het gebruik van bacteriën om elementen uit planetair gesteenten en asteroïden te halen en verder te gebruiken. Dit onderzoek was er daarom op gericht om de interactie van de bacterie *Cupriavidus metallidurans* CH34 met basalt, een vulkanisch maan-analoog gesteente, te onderzoeken. De bacteriële aanwezigheid op basalt alsook het effect van verschillende soorten basalt op groei van de bacteriën werd bestudeerd. Daarnaast werd de bacteriële overleving en de mogelijke invloed van de ruimte omgeving (bv. verlies aan zwaartekracht) op deze interacties bepaald.

Allereerst werden de verschillende bacteriële gemeenschappen aanwezig op elkaar volgende, en dus oudere, basaltafzettingen bestudeerd. Zo kon de relatie tussen de bacteriële aanwezigheid, basalt compositie en verwerking van de basalt en eventuele kolonisatie bepaald worden. Hierbij werd gezien dat pionierssoorten zoals *Acidobacteria* en *Actinobacteria* de eerste zijn die deze extreme omgeving zouden kunnen koloniseren en dat misschien N₂-fixeerders zoals *Planctomycetaceae*, *Rhizobiales Sphingobacteriales* en *Acetobacteraceae*, *Clostridium*, *Chitinophagaceae* en heterotrofen zoals *Ralstonia* verantwoordelijk zouden zijn voor de verschuiving tussen de oudere en jongere afzettingen, waardoor rhizosfeer en bodembacteriën deze omgeving verder kunnen koloniseren.

Vervolgens werd de impact van verschillende basalt samenstellingen onderzocht voor vrijgave van elementen en ondersteuning van bacteriële groei. Onze resultaten tonen duidelijk aan dat verschillende soorten basalt, verschillende hoeveelheden elementen

in het medium vrijgeven, zoals bv. ijzer, calcium, magnesium en fosfor. De afgifte van deze elementen beïnvloedt de groeisnelheid en eindconcentratie van deze bacteriën en bv. alle geteste basalten kunnen de groei in ijzer limiterend media herstellen, maar niet in magnesium limiterend medium. Dit was echter niet gecorreleerd met de elementaire compositie van basalt, behalve voor magnesium.

Daarnaast werden de effecten van langdurige opslag op een bacterieel inoculum en zijn interactie met basalt gesteente bestudeerd. We konden aantonen dat basalt een positief effect heeft op de bacteriële overleving door elementen zoals natrium, kalium en fosfaat in het water vrij te zetten, waardoor nadelige effecten van inoculumopslag kunnen worden verminderd. Cellen in de aanwezigheid van basalt zijn meer cultiveerbaar, bevatten hogere hoeveelheden ATP en minder cellen zijn gepermeabiliseerd en cellen bevatten minder PHB. Cellen vormden ook een biofilm op basalt waardoor de cellen beter beschermd zijn in deze limiterende omstandigheden. Ruimte omstandigheden hadden ook een positief effect op overleving, en cellen zijn meer cultiveerbaar, bevatten meer ATP en meer cellen zijn intact en actief. In deze omstandigheden werd er ook minder biofilm gevormd en zetten de cellen koper vrij in het water.

Daarnaast werd flow cytometrie gebruikt als een techniek om verschillende bacteriële fysiologische parameters in te schatten en de heterogeniteit van een bacteriële populatie te beoordelen. Hierbij werd gezien dat de rode SYTO-kleuringen konden worden gebruikt als “counter” kleuringen in combinatie met andere functionele kleuringen. De efficiëntie van de verschillende combinaties verschilde tussen de geteste organismen en bevestigde dat optimalisatie nodig is om nauwkeurige functionele metingen uit te voeren. Bovendien beïnvloeden sommige kleurstofcombinaties, alsook stabiliteit van deze combinaties, de resultaten, en juiste controles zijn hiervoor belangrijk in het onderbouwen en bevestigen van de verkregen resultaten.

CV

CURRICULUM VITAE

Education

PhD candidate

2013-present

"Extractions of elements from minerals with bacteria in space conditions"

Supervisors: Prof. Nico Boon (UGhent), Dr. Natalie Leys (SCK•CEN), Rob Van Houdt (SCK•CEN)

Center of Microbial Ecology and Technology (CMET), Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, University Of Ghent in collaboration with the Microbiology Unit at the Belgian Nuclear Research Center (SCK•CEN)

Master of Science in the Bioengineering Sciences:

Cell and Gene Biotechnology

2009-2013

Minor in Industrial Microbiology

Thesis: *"Effects of low pH on the growth of E. coli"*

Supervisors: Prof. Chris Michiels and Prof. Abraham Aertsen

University of Leuven

Bachelor of Life Sciences

2006-2009

University of Hasselt

Peer-reviewed publications

- Byloos, B. Van Houdt, R., O. Boon, N. Leys, N. The impact of survival conditions on the interaction and cultivability of Cupriavidus metallidurans CH34 with basalt, a volcanic moon analog rock. In preparation
- Byloos, B. Van Houdt, R. Mysara, Monsieurs, P. Boon, N. Leys, N. Chronosequencing the bacterial communities on lava deposits that make them habitable. In preparation
- Byloos, B. Maan, H. Van Houdt, R., O. Boon, N. Leys, N. The ability of basalt to leach nutrients and support growth of Cupriavidus metallidurans CH34 depends on basalt composition and element release. Accepted in Geomicrobiology Journal
- Loudon, C. Nicholson, N. Finster, K. Leys, N. Byloos, B. Van Houdt, R. Rettberg, P. Moller, R. Fuchs, F. Demets, R. Vukich, M. Mariani, A. Cockell, C. (2017). BioRock: New Experiments and Hardware to Investigate Microbe-Mineral Interactions in Space. International Journal of Astrobiology. 1-11
- Byloos, B. Coninx, I. Van Hoey, O. Cockell, C. Nicholson, N. Ilyin, V. Van Houdt, R. Boon, N. Leys, N. The impact of space flight on survival and interaction of Cupriavidus metallidurans CH34 with basalt, a volcanic moon analog rock. Frontiers in Microbiology 8, 671.
- Buysschaert, B. and Byloos, B., Van Houdt, R. Leys, N. Boon, N. Re-evaluating multicolor flow cytometry to assess microbial viability. (2016), Applied Microbiology and Biotechnology, 100(21), 9037-9051.
- Bryce, C. Le Bihan, T. Martin, S. Harrison, J. Bush, T. Spears, B. Moore, A. Leys, N. Byloos, B. Cockell, C. (2015), Rock geochemistry induces stress and starvation responses in the bacterial proteome. Environmental Microbiology, 18: 1110–1121.

Oral Presentations

- Byloos, B. Van Houdt, R. Boon, N. Leys, N. Potential of Cupriavidus metallidurans CH34 for in situ resource utilization from basalt by determining the molecular microbe-mineral interactions. 40th COSPAR Scientific Assembly, Moscow, Russia, 2-10 August 2014.
- Byloos, B. Van Houdt, R. Boon, N. Leys, N. Potential of Cupriavidus metallidurans CH34 for in situ resource utilization from basalt by determining the molecular microbe-mineral interactions. Part of the Space Factor contest. EANA, Edinburgh, UK, 13-16 October 2014.
- Byloos, B. Van Houdt, R. Leys, N. Outcome of the BIOROCK precursor experiment on Foton M4. BIOROCK Meeting, ESTEC, Noordwijk, Netherlands, 16 January 2015.
- Byloos, B. Van Houdt, R. Boon, N. Leys, N. The molecular mechanisms behind microbe-mineral interactions, on Earth and in Space. GESE meeting. Iglesias, Sardinia, 25-28 May 2015.

- Childers, D. Nicholson, N. Fuchs, F. Byloos, B. Van Houdt, R. Finster, K. Möller, R. Leys, N. Cockell, C. BIOROCK: An International Space Station Mission to Investigate Bacterial Biofilm Formation and Mineral Extraction from Natural Substrates in Micro- and Simulated Gravity. EANA, ESTEC Noordwijk, Netherlands, 6-9 October 2015.

Poster Presentations

- Byloos, B. Van Houdt, R. Boon, N. Leys, N. Potential of *Cupriavidus metallidurans* CH34 for biomining from lunar-like basalt by determining the molecular mechanisms behind microbe-mineral interactions. AbGradE Meeting, Edinburgh, UK, 9-10 October 2014.
- Bryce, C. Martin, S. Le Bhian, T. Bush, T. Spears, B. Moore, A. Harrison, J. Leys, N. Byloos, B. Cockell, C. Rock Chemistry drives stress and starvation response in the bacterial proteome. EANA, Edinburgh, UK, 13-16 October 2014.
- Byloos, B. Van Houdt, R. Boon, N. Leys, N. The molecular mechanisms behind microbe-mineral interactions, on Earth and in Space. Day of the PhD's, Mol, Belgium, 23 October 2014.
- Byloos, B. Van Houdt, R. Boon, N. Leys, N. The molecular mechanisms behind microbe-mineral interactions, on Earth and in Space. BSM meeting, Brussels, Belgium, 18 November 2014.
- Byloos, B. and Buysschaert, B. Lerno, S. Props, R. Van Houdt, R. Leys, N. Boon, N. Multicolor flow cytometry to determine bacterial viability. How dead is dead conference. Zurich, Switzerland, 21-22 May 2015.
- Byloos, B. Van Houdt, R. Cockell, C. Ilyin, V. Boon, N. Leys, N. The molecular mechanisms behind microbe-mineral interactions, on Earth and in Space. FEMS Meeting, Maastricht, Netherlands, 7-11 June 2015.
- Samuels, T. and Schwendner, P. Baqué, M. Byloos, B. Fox-Powell, M. Journaux, M. Mora, M. Musilova, M. Nauny, P. Noack, L. Perras, A. Serrano, P. Simoncine, E. Stevans, A. Taubner, S. Verseux, C. AbGradE: Connecting the next generation of early-career astrobiologists. AbSciCon meeting. Chicago, USA, 15-19 June 2015.
- Byloos, B. Van Houdt, R. Mysara, M. Vilhelmsson, O. Boon, N. Leys, N. Bacterial communities on lava deposits of different ages from Krafla, Iceland. EANA, ESTEC Noordwijk, Netherlands, 6-9 October 2015.
- Byloos, B; Van Wallegghem, M. Nicholson, N. Van Houdt, R. Ilyin, V. Cockell, C. Boon, N. Leys, N. The interactions of the bacterium *Cupriavidus metallidurans* CH34 with basalt rock, on Earth and in Space. Summer school 'Biosignatures and the Search for Life on Mars', Iceland, 4 - 16 July 2016.
- Byloos, B; Nicholson, N. Van Houdt, R. Ilyin, V. Cockell, C. Boon, N. Leys, N. The interactions of the bacterium *Cupriavidus metallidurans* CH34 with basalt rock, on Earth and in Space. Summer school 'Biosignatures and the Search for Life on Mars', Iceland, 4 - 16 July 2016.
- Byloos, B; Nicholson, N. Van Houdt, R. Ilyin, V. Cockell, C. Boon, N. Leys, N. The interactions of the bacterium *Cupriavidus metallidurans* CH34 with basalt rock, on Earth and in Space. MELISSA Mini-Symposium and Master Class. Antwerp, Belgium. 30th of March 2017.

Awards and grants

- Poster Award MELISSA Mini-Symposium and Master Class. 30th of March 2017, Antwerp, Belgium.
- Travel grant COST Action "Origins and Evolution of Life on Earth and in the Universe" to attend the Summer School: 'Biosignatures and the Search for Life on Mars'. 3-17 July 2016, Iceland.
- Early Career Scientist Grant and Award of the Federation of European Microbiologists (FEMS) to participate to the FEMS conference, 7-11 June 2015. Maastricht, Netherlands.
- Poster award at EANA conference: Potential of *Cupriavidus metallidurans* CH34 for in situ resource utilization from basalt by determining the molecular microbe-mineral interactions. 13-16 October 2014. Edinburgh, UK.
- Travel Grant to participate to the 40th COSPAR Scientific Assembly. 2-10 August 2014. Moscow, Russia.
- Travel grant to participate to the Erasmus training program on 'Arctic Microbiology' to the University of Akureyri, Iceland, 14-28 June 2014.

Teaching

- Effects of space conditions on bacterial cells: application of a flow 'cell viability' cytometry technique. Printemps des Sciences 24/03/2017. SCK-CEN, Mol.
- Effects of space conditions on bacterial cells. Summer School in Radiobiology. 8- 19/08/2016. SCK-CEN, Mol.
- MELISSA: the life support system in Space. Master after Master in Space. 18-11-2015. SCK-CEN, Mol.
- Guided one master student. Physiology and growth of *Cupriavidus metallidurans* CH34 in the presence of basalt, a lunar-like rock, on Earth and in Space. September 2016-June 2017.

Courses attended

- Erasmus Mundus training program on 'Arctic Microbiology' at the University of Akureyri, Iceland, 14-28 June 2014. Organized by the Doctoral Schools of UGhent (B), Reading (UK) and Jacobs University Bremen (DE). Expedition to volcanic environments in Iceland and training plus education in field work and sampling of glaciers, geothermal zones, desert zones, glaciers and rivers for microbiology analysis.
- Summer School: Biosignatures and the Search for Life on Mars in Iceland, 4 - 16 July 2016. Subject of the search for life in different environments, both on 'early Earth', below Earth's surface and on Mars, organized by the Nordic Network of Astrobiology, the European Astrobiology Campus, and the EU COST Action "Origins and Evolution of Life on Earth and in the Universe".
- Course of Radiation Protection, 18-22 October 2013, SCK•CEN
- Course of Mixture Toxicity in the frame of the STAR project, 27-30 January 2014, SCK•CEN
- Scientific Writing and Speaking Course, 4 days in March and April, 2014, SCK•CEN
- Microbial Evolution: theory, simulation and experiment, 4 and 5th of May 2015, Leuven, Belgium
- Advanced Academic English: Conference Skills. 16 February to 3rd of March 2016. UGhent, Gent, Belgium
- Elixir workshop: Introduction to Genomics Analysis. 7 and 8 november 2016. BELSPO, Brussels, Belgium
- Applying for a postdoctoral job, 3 days in January and February 2017. UGent, Gent Belgium

CV

Dankwoord

Dankwoord

Dit is op zijn zachts uitgedrukt het meest emotionele en traan-inducerende deel van mijn thesis om te schrijven en woorden schieten te kort, om al de mensen te bedanken die mij de laatste jaren gesteund hebben. Er is maar één goed woord om het allemaal samen te vatten: “een expeditie” en heel wat mensen zijn verantwoordelijk voor het slagen ervan!

Het belangrijkste deel van deze ‘expeditie’, de navigatoren, mijn drie promotoren/inspiraties/mentoren/...: Natalie, Rob en Nico. Eerst en vooral, Natalie, bedankt om zoveel werk en tijd vrij te maken en mij het vertrouwen te geven zodat ik kon groeien als ‘onderzoeker’. De naïviteit en nieuwsgierigheid die ik in het begin had (een PHB mutant maken in 2 weken, geen probleem!) heb ik leren vertalen in realistische plannen met soms onverwachte bestemmingen (het FOTON vlucht experiment). “Rob, zou ik je iets mogen vragen?”. Het werd zo een beetje mijn slagzin als ik je opnieuw kwam storen, maar dat was dan ook omdat ik wist dat ik altijd bij je terecht kon voor eender welke vraag die ik ook maar moest hebben. Dat kon gaan van: hoe ga ik om met moeilijke reviewers (“Wacht tot morgen voor je je antwoord formuleert, laat het eerst even rusten”) tot hoe werkt die techniek? (“Ik toon het je wel”),...Beiden hebben jullie me geholpen om kritisch naar mijn data te kijken en sluitende oplossingen/experimenten/...op te zetten tot het hele verhaal klopte. Nico, ik keek altijd enorm uit naar onze discussies, omdat ik daarna altijd nieuwe ideeën kreeg en andere insteken had, om naar mijn onderzoek te kijken. Alle drie hebben jullie mij met raad en daad bijgestaan zodat deze expeditie tot een goed einde kon gebracht worden en kreeg ik alle steun als ik opnieuw het zotte plan had opgevat om opnieuw naar IJsland te vertrekken,... Het was een privilege om deel uit te maken van twee fantastische labo’s en te kunnen bijdragen aan het fantastische ruimte en niet-ruimte onderzoek dat hier gebeurt! DankUwel!

I would also like to thank my jurymembers, Prof. Monica Höfte, Prof. Geelen, Prof. Hennebel, Prof. Carlier, Prof. Cnudde, Prof. Swennen en Prof. Finster, for being part of my jury and for their very constructive comments and discussions during my private defense, it really helped me to enhance my thesis and make the results of this expedition more clear.

I would also really like to thank all the people involved in the BIOROCK project, who were responsible for equipping this expedition, your continuous support and collaboration has really pushed it forward and I am hoping for a good future space flight experiment. Also all the people of AbGradE, the Icelandic expeditions (the ICE-landers and the Wolfpack); you guys were fantastic and I learned so much (both astrobiology and non-astrobiology related! ☺).

All the people of the microbiology unit at SCK, your support has been invaluable for my expedition. You transformed the, at first, extreme environment of a lab into a habitable one, helping me to find my place and make it all practically possible. In addition, I would also like to thank the people of the radiobiology unit, that supported me through the project. Next, our secretary Betty, to tackle and guide me through all the flying papers, booklets, traincards,... Also, Jorden, Jean, Axel, Mieke, Ben and Jef of the other groups at SCK and NIRAS, this work couldn't have been done without your practical help to prepare and analyze the (in total ± 50 kg of basalt) rock or do a new element analysis!

Next to the lab at Mol, I spend a small (but hopefully productive) part of the expedition at the lab in Gent. Thank you to all the people at CMET: Ruben, Benjamin, Jo, FM, Sylvia, Regine, Christine and Sarah. Coming to Gent was an expedition on itself but thank you for always letting me feel welcome. Thank you to the people of the ecology cluster, your advice was invaluable for my research. Ruben, de nachten op SCK waar je bijna vergroeid was met de FCM, die ga ik niet meer vergeten. Benjamin, het waren echt een paar geweldige maanden in het labo in Gent, voor ons flow cytometrie artikel, en ik kan mij niet inbeelden hoe ik ooit alleen een eerste artikel had kunnen schrijven!

Next to the lab, I was also part of our Morpheus PhD group at SCK and would like to thank all the PhD, also the past ones (Annelies, Tine, Marlies, Ellina, Kevin (yeah, you too!), Sandra, Hanène and Joachim), you guys totally rock to the moon and back! Thank you for keeping me straight on my path when I flew off course! ☺ Next, the Mariogold movie aficionados group, which share my love for everything film! ('That is a bingo!'). Merel, wij zijn deze expeditie als drie musketiers begonnen en ik kon bij jou altijd terecht voor de soms broodnodige stoom aflatende gesprekken. Naast het labo, hebben we ook een deel van Europa samen verkent, zowel voor/op/na het begin van een nieuw jaar, and we are aiming for the stars! ☺

Mieke, Nicole and Niels, you guys rocked as office mates! Niels, dees is echt niet te gelove, ma we hemme dat toch mer wee schoun gedon! Onze roadtrip is gewoon “the pimped out adventure of Bno and Neil” en ik ga je missen as work-husband! Mieke, ik besef opeens dat ik echt nog zo groen achter mijn oren zag, toen je bij mij op de bureau kwam en ik kan echt niet begrijpen hoe je ooit met mij hebt uitgehouden. Nicole, I wish you all the best there in Trumpstate! Learning from you was a great privilege and I hope that I have returned the favor with my knowledge about (Belgian/Christmas/woman) beer and Belgian radio stations! Anu, Mona and Akshaya, we always had great discussions and it was great having you in our office!

Bjorn, of misschien beter Dr. Baselet, zoals je al zei, als ik je moet bedanken voor alles wat je voor mij gedaan/betekent hebt, heb ik meer dan deze 260 pagina's nodig. Maar toch een kleine bloemlezing; de laatste maanden waren enorm stresserend maar je was de beste steun die iemand kan wensen (We gaan zo hard borden kapot gooien als het allemaal achter de rug is, see it settled! ☺). Het begon allemaal vier jaar geleden, aangezien we dezelve initialen delen, maar groeide uit tot road trips (USA and Canada express, Wenen, laten we aub zwijgen over Zürich....), concerten, opera's, musicals, film avonden/nachten/marathons, B'tjes go loco (met natuurlijk Jolien, mijn tentbuddy for life! ☺). Ik hoop dan ook dat we nog fantastische avonturen mogen beleven, en wens je nog alle succes!

Daarnaast wil ik ook al de rest van mijn vrienden bedanken; jullie gaven me de ontspanning en afleiding die ik nodig had om het allemaal te kunnen laten rusten, ik kan me er dan ook geen betere inbeelden! Suzanne, de laatste jaren zijn we samen al door heel wat wateren gevaren, en je bent voor mij de zus die ik nooit gehad heb. Je 'Suzanne stijl' is fantastisch (zowel in het schrijven als gewoon in je 'zijn' ;)) en ik hoop dat je die stijl nooit verliest!

Als laatste, mijn familie, dit PhD begint en eindigt eigenlijk allemaal bij jullie, zonder jullie steun zou dit nooit gelukt zijn: mijn lieve grootouders; bomma en bompa; jullie zijn nog altijd als mijn tweede paar ouders en hebben me van kleins af met zo veel liefde omringd! Anita, Geert, Robbe en Anne, jullie hebben me altijd gesteund en ik hoop dat ik eindelijk eens iets terug kan doen. Vava en moemoe, al de zussen en nichten, het is echt super leuk om deel uit te maken van zo een grote en geweldige familie! Sabrina, ik ga de SCK onderonsjes missen! ☺

Liefste mama en papa, jullie zijn de beste en liefste ouders die iemand kan hebben. Ik zie jullie graag en ben zo dankbaar voor de kansen, steun en die liefde die jullie me gegeven hebben. Het is niet altijd eenvoudig geweest; voor en tijdens mijn studies aan de unief, en nu vier jaar doctoraat. Ik ben ook niet altijd de eenvoudigste, en een echte keikop, maar met jullie steun kom ik er altijd door en met jullie steun weet ik ook dat altijd alles goed komt.

Pfffhieuuw, I think I Leo (Dicaprio)'it! :p

And please one last time: Give it up for basalt, it rocks! (I know it is stupid)

Thank you!